Rhizosphere microbiome assembly and function in the Brassicaceae

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Dedication

I dedicate this thesis to my parents, Karen Beltrami and Michael Monaghan. Their support throughout the course of my PhD was invaluable to me. I will always be grateful for their love and support then, now and in the future.

Acknowledgements

I would like to acknowledge the many people who have helped me throughout my PhD. Their assistance enabled me to carry out the many experiments I undertook and ultimately the completion of my thesis.

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Finally, I would like to acknowledge the unending support of my parents, who this thesis is dedicated to. Their love, support and advice were invaluable throughout this process.

Declaration

I declare that the work presented in this thesis was conducted by myself under the direct supervision of Professor Gary Bending and Professor Murray Grant, with the exception of instances where the contribution of others has been specifically acknowledged. None of the work presented in this thesis has previously been submitted for any other degree.

Ellis Beltrami Monaghan

List of abbreviations

(%)	Percentage
(c)	Class
(f)	Family
(g)	Genus
(k)	Kingdom
(o)	Order
(p)	Phylum
(s)	Species
Aao3	Abscisic acid deficient *A. thaliana* (KO Arabidopsis aldehyde III)
ABA	Abscisic acid
Aba	Abscisic acid hypersensitive mutant (protein phosphatase 2C mutant)
AHDB	Agriculture and Horticulture Development Board
AM	Arbuscular Mycorrhiza
ANOSIM	Analysis of Similarity
ASV	Amplicon Sequence Variant
BLAST	Basic Local Alignment Search Tool
Can	Canard
Cat	Catana
cm	Centimetre
Col0	Wild-type *A. thaliana* (Columbia ecotype)
Com	Compass
DEFRA	Department for Environmental, Food and Rural Affairs
DNA	Deoxyribonucleic acid
FAO	Food and Agricultural Organisation of the United Nations
FAOSTAT	Food and Agricultural Organisation of the United Nations Statistics Department
FastSpar	C++ implementation of the SparCC algorithm
g	Gram
ha	Hectares
hg	Heptagrams
indval	Indicator Value
ITS	Internal Transcribed Spacer
JA	Jasmonic acid
kg	Kilogram
L	Litres
m	Metre
mg	Milligram
min	Minute
ml	Millilitres

N	Number
NCBI	National Centre for Biotechnology Information
NMDS	Non-Metric Multidimensional Scaling
ºC	Degrees Celcius
OSR	Oilseed Rape
OTU	Operational Taxonomic Unit
PAST	Paleontological Statistics Software Package for Education and Data Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational analysis of variance
pH	Potential hydrogen
QIIME	Quantitative Insights into Microbial Ecology
Rhz	Rhizosphere Soil
Ro	Root
rpm	Rotations per minute
rRNA	Ribosomal Ribonucleic acid
SA	Salicylic acid
SDW	Sterile Deionised Water
Sid2	Salicylic acid deficient *A. thaliana* (KO isochorismate synthase)
SIMPER	similarity percentages breakdown procedure
SparCC	Sparse Correlations for Compositional data
STDEV	Standard Deviation
t/ha	Tonnes per Hectare
Tem	Temple
UK	United Kingdom
Wel.	Wellesbourne
ADONIS	Permutational analysis of variance function used in R software (vegan package)

Summary

The rhizosphere microbiome is of great importance to plant health and function being described as the plants 'second genome' and an essential component in the host plants biology, ecology, and evolution. Subsequently, understanding the assembly and function of rhizosphere microbiomes can be seen as essential to the development of modern agriculture with the aim of developing microbial based agricultural tools. However, a systematic understanding of how the microbiota assembles and functions as a whole in relation to the plant host is not fully developed. Therefore, this thesis investigated the assembly and functionality of rhizosphere microbiomes in two important Brassicaceae plant species, *Brassica napus* and *Arabidopsis thaliana* to develop understanding of rhizosphere microbiome assembly and functionality. Analysis of rhizosphere microbiome assembly in response to plant genetic diversity was assessed in the key crop species *B. napus*. The rhizosphere soil and root microbiomes of four *B. napus* genotypes (or cultivars) with distinct root architecture and exudate profiles were assessed in a large-scale field trial. This investigation indicated that these genotypes did not alter microbiome composition at the community level. However, indicator species analysis and inter-taxon community network analysis identified significant indicator taxa for each genotype and genotype specific network architectures. In addition, the diversity of root and rhizosphere soil communities were found to be significant explanatory variables of biomass and yield in certain genotypes. Together these results indicate that genotypic influences on the rhizosphere microbiome are complex and can be nuanced. Functionality of the fungal rhizosphere microbiome of *B. napus* was also investigated in this thesis. A collection of fungal isolates from root and rhizosphere soil samples of *B. napus* were successfully isolated and utilised in plant – microbe co-incubation studies. This collection included isolates representative of many taxa present in the UK *B. napus* rhizosphere mycobiome and included, taxa correlated positively and negatively with *B. napus* yield and, several potentially pathogenic and endophytic taxa. Six taxa with deleterious impacts on *B. napus* seedling biomass were identified. Taxa correlated with yield were also successfully screened, however, no significant impacts on biomass were identified. This indicated that their potential influence on yield may occur through other means. Finally, analysis of rhizosphere mycobiome assembly in

response to *A. thaliana* genotypes with immune phytohormone production and sensitivity mutations was carried out. Wild-type Col0 and three immune phytohormone mutant *A. thaliana* genotypes with either, hypersensitivity to Abscisic acid, deficiency in Abscisic acid production and deficiency in Salicylic acid production were analysed. This thesis presents the first research regarding fungal microbiome assembly in a Salicylic acid deficient plant mutant and the first research on any microbiome assembly in Abscisic Acid mutant plants. Immune phytohormone genetic diversity was not found to alter community diversity indices. However, significant enrichments of Basidiomycota were identified in Abscisic acid mutants. In addition, immune phytohormone deficient genotypes were not found to form 'deleterious' root mycobiomes. In addition, microbiome transfer using mutant conditioned soils were not found to alter community composition, diversity, and plant biomass in subsequent Wild-type *A. thaliana*. Together the results presented in this thesis develop our understanding of plant – microbiome assembly and functionality in the Brassicaceae and plants in general.

1 General Introduction

1.1 Oilseed rape: a crop of global importance

1.1.1 Oilseed rape (*Brassica napus*)

Oilseed rape (OSR), taxonomic name: *Brassica napus*, is an agricultural crop cultivated across the globe for the production of VLCFAs (Very Long Chain Fatty Acids) which are used for industrial, biotechnological and nutritional applications such animal feedstock and biofuel production substrates (McVetty et al., 2016). From an agronomic perspective, OSR is a key break crop with wheat providing an over winter cash crop which boosts subsequent wheat disease resistance and yield (Angus et al., 1991). As of 2019 Global trends in the production of OSR have seen increases in yield and cultivation area from the 1960's onwards (**Figure 1.1**), with OSR becoming the second most produced oilseed crop after soybean from the early 2000's (Carré and Pouzet, 2014, FAO, 2019a). This has mainly been due to the introduction

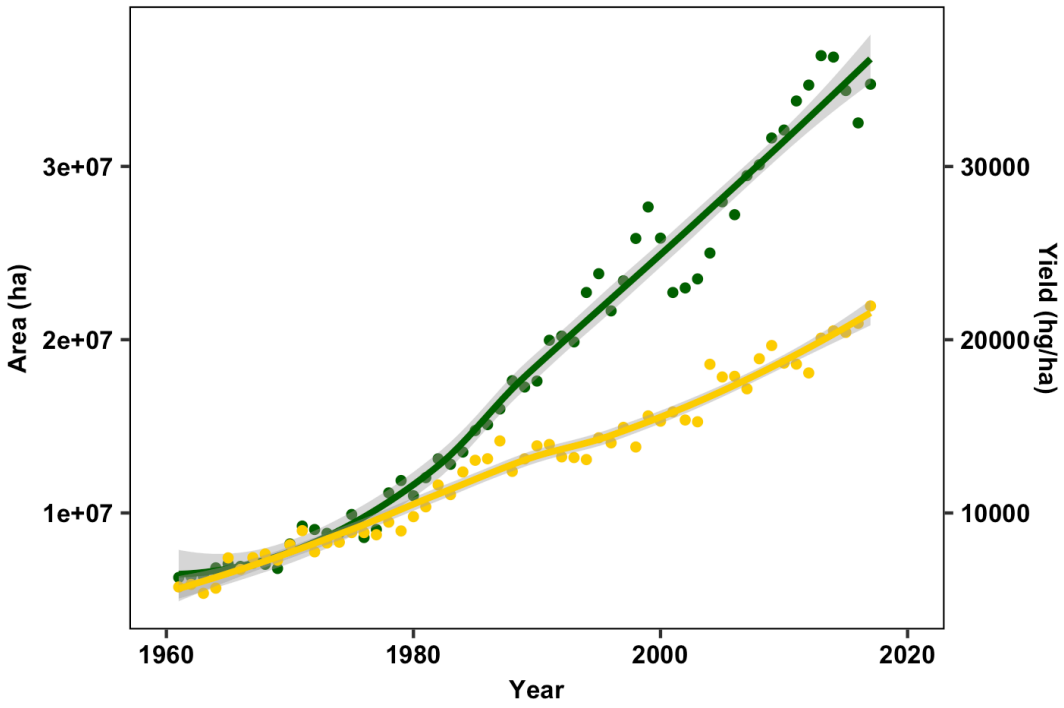

Figure 1.1 Global trends in OSR production from 1961 to 2017*. Green dots are representative of total global OSR area (ha) with yellow dots representative of total global yield (hg/ha). Trendlines represent smoothed mean of respective data. Data obtained from FAOSTAT (FAO, 2019).*

of high yielding varieties, hybrid OSR lines and low erucic acid/ glucosinolate lines which are suitable for human consumption.

OSR is a member of the Brassicaceae family along with the scientifically important model organism *Arabidopsis thaliana*. It is a member of the Brassica genus which includes several wild species and five other important crop species (*Brassica carinata*, *Brassica juncea*, *Brassica nigra*, *Brassica oleracea* and *Brassica rapa*). Including OSR (*B. napus*), these Brassica crop species form the Triangle of U (**Figure 1.2**) (Nagaharu, 1935). *B. nigra, B. oleracea* and *B. rapa* are diploid species which occurred from a polyploidy event between 7.9 and 14.6 million years ago. *B. carinata, B. juncea* and *B. napus* are amphidiploid species, resulting from hybridisations of the three diploid Brassica species (**Figure 1.2**)(Nagaharu, 1935).

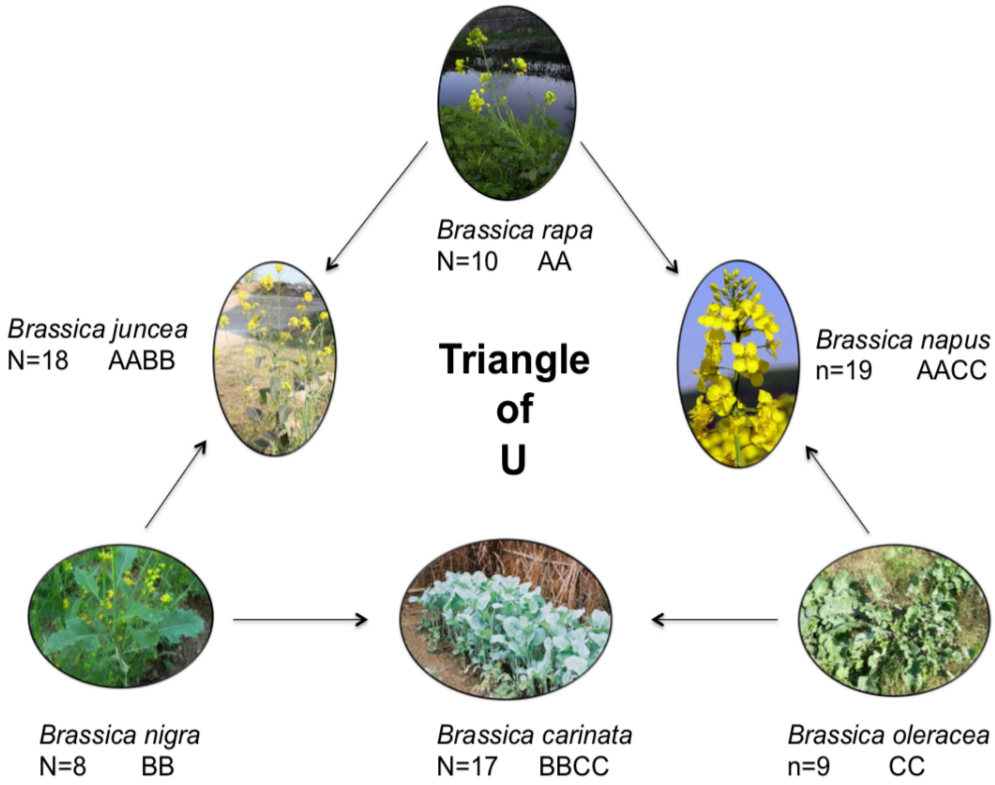

Figure 1.2 Triangle of U representing genetic relationships of the Brassica genus crops. *n = number of chromosomes and letter denotes genome classification. Taken from (Hale, 2017).*

1.1.2 Challenges in OSR cultivation: crop security, yield stagnation and soilborne microorganisms

There are several challenges to the cultivation OSR and the future development of healthy and productive OSR crops despite gains made due to the introduction of high yielding varieties. These include a range of factors such as; pathogenic microorganisms and animal pests (AHDB, 2018, Zheng et al., 2020), the slowing of yield gain growth in Europe and Australia (Zheng et al., 2020), and the yield stagnation phenomena which has occurred in the United Kingdom since 1985 (**Figure 1.3**) (FAO, 2019b, Rondanini et al., 2012).

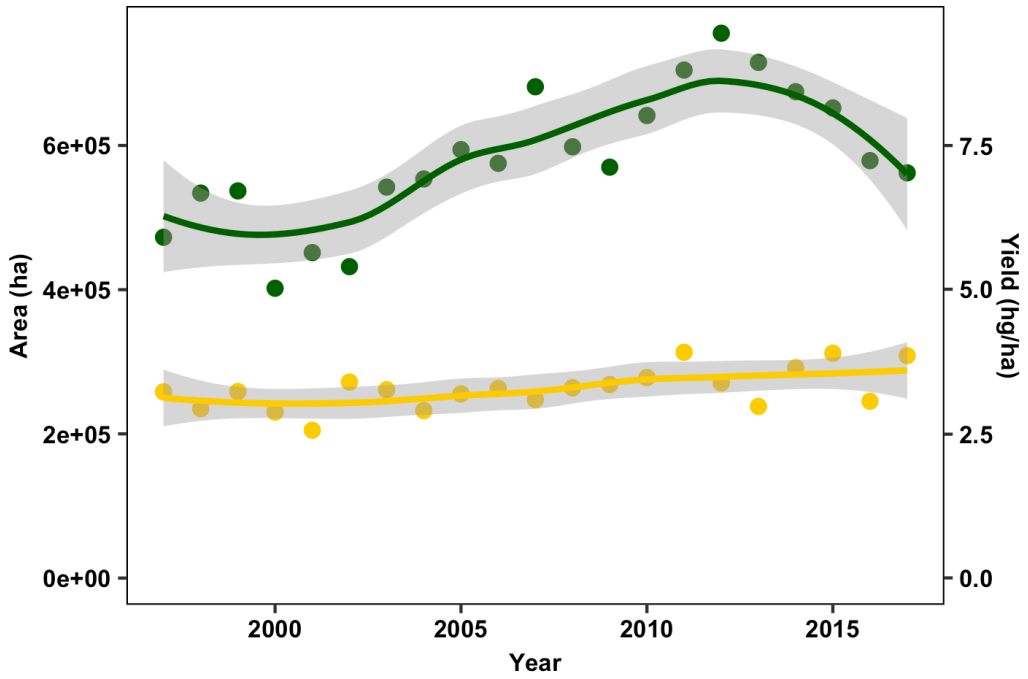

Figure 1.3 Trends in UK OSR production from 1997 to 2017. *Green dots are representative of total UK OSR area (ha) with yellow dots representative of total UK yield (hg/ha). Trendlines represent smoothed mean of respective data. Data obtained from FAOSTAT (FAO,2019).*

A recent review of the major diseases and pests which affect OSR crops across the globe have identified 32 insect pests, 16 microorganism borne diseases and several nematode and slug species of major impact (Zheng et al., 2020). These biotic factors were implicated in the reduction of yield gains identified by this study in Europe and Australia. Insect pests of major concern varied depending on global region, however, microbial diseases of major concern were more consistently identified across all global regions with three major diseases and their causative agents identified (Sclerotinia stem rot (*Sclerotinia sclerotiorum*), Phoma stem canker (*Leptosphaeria maculans* and

Leptosphaeria biglobosa) and clubroot (*Plasmodiophora brassicae*)). All of these pathogens are associated with significant disease severity and reduction in final yields of OSR (Zheng et al., 2020).

Many pathogenic taxa which affect OSR, including those identified by Zheng et al. (2020), are soilborne. *S. sclerotiorum* and *P. brassicae* can survive in infected soils for up to four and fifteen years respectively. *S. sclerotiorum* is of particular concern given that it can infect over 400 plant species (Mizubuti, 2019). Long persistence in soils and broad host range of pathogenic taxa is concerning in OSR given its use as a break crop (Angus et al., 1991) and the short rotation strategies employed in countries such as the UK which are already associated with yield penalties (Knight et al., 2012) (**Figure 1.4**). Shortened rotations and continual monoculture has also been shown to cause the successive build-up of *Olpidium brassicae* & *Pyrenochaeta lycopersici* in OSR roots in the UK (Hilton et al., 2013, Hilton et al., 2021). These fungal taxa are not listed as major diseases of OSR, however, Hilton et al. (2013), identified significant negative impacts on seed quantity and quality in a glasshouse study of infected OSR, implicating their potential importance in yield stagnation. This was further supported in Hilton et al. (2021), in which a landscape scale study identified a significant correlation between shortened rotations, *O. brassicae* (and other pathotrophic fungi) and lower yields. In addition to

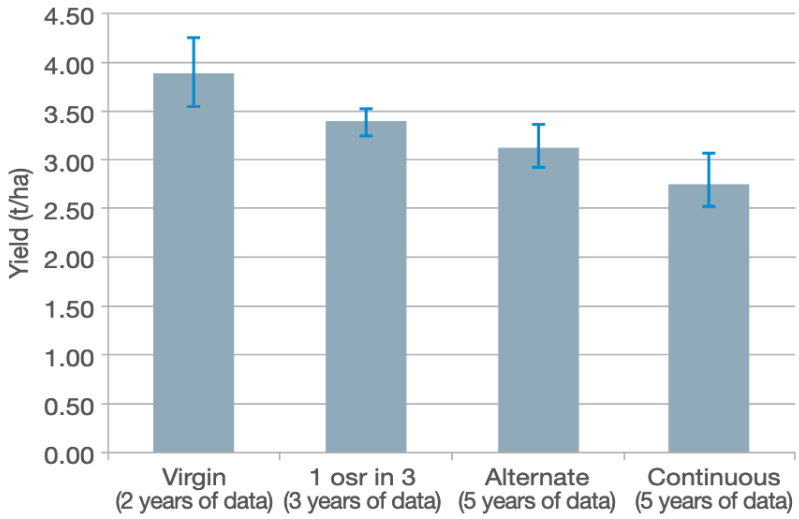

Figure 1.4 Yield penalties associated with various OSR - wheat rotations. *Data is representative of mean OSR yield achieved in different agricultural rotations with wheat. Taken from (AHDB,2018).*

these taxa, the Agricultural and Horticultural Development Board (AHDB) of the UK identifies two other soilborne pathogens of note namely *Rhizoctonia solani* and *Verticillium longisporum*. The AHDB notes that *R. solani* infection is associated with OSR establishment losses of 17 – 65 % with subsequent yield losses possible at plant densities below 25 plants/m^2 (AHDB, 2021). Regarding *V. longisporum*, it notes that it is a 'persistent soilborne disease' with yield reductions in the range of 3 – 34 % recorded (AHDB, 2021).

As many of the diseases which affect OSR security and yield are soilborne, this indicates that the soil microbiota is key to OSR health and the development of crop security and yield boosting strategies. Indeed, the root and rhizosphere soil microbiomes (i.e. plant tissue in direct contact with the soil and the area of soil influenced by the plant, see section 1.2) of plants are known to harbour a diverse range of microbiota interacting with the host plant in mutually beneficial and deleterious ways which are essential to the host plants health and development (Berendsen et al., 2012). However, our understanding of how the rhizosphere microbiome assembles in plants, and subsequently how it may be harnessed for beneficial traits is still in its infancy.

1.2 Rhizosphere microbiomes

1.2.1 Rhizosphere microbiome assembly

Soils are heterogenous and highly complex environments in which diverse communities of microorganisms including; bacteria, fungi, protists, viruses, archaea, and nematodes inhabit (Fierer, 2017). It has been noted as early as the 19th century that soil microorganisms are key to nutrient cycling in soils and can form close

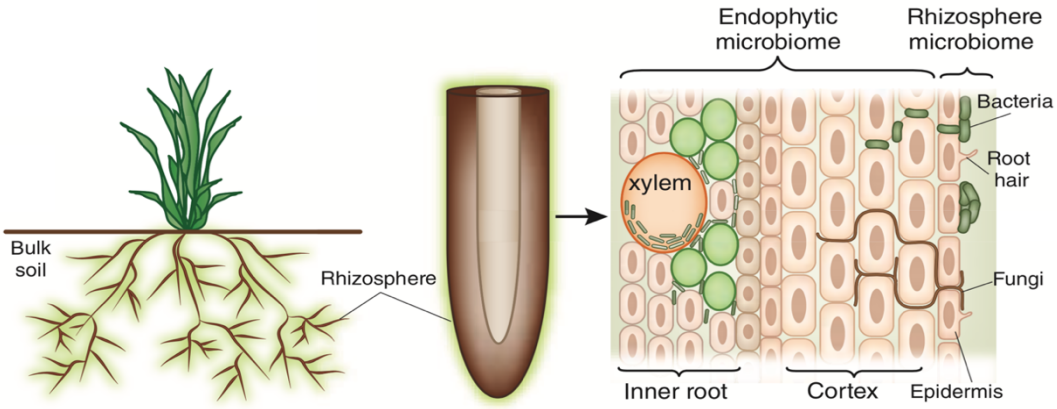

Figure 1.5 Schematic diagram of the rhizosphere microbiome detailing its location relative to plant roots. *Taken from (Hirsch & Mauchline, 2012).*

complex relationships with plants at the root-soil interface (Hirsch and Mauchline, 2012). Microorganisms which live in close association with plants at the root – soil interface constitute the rhizosphere microbiome, which was first defined by Lorenz Hiltner in 1904 as 'soil influenced by roots' (Hartmann et al., 2008). Since, the rhizosphere microbiome has been seen as a key component in plant biology and health (Berendsen et al., 2012, Hirsch and Mauchline, 2012).

The rhizosphere comprises of three main compartments; the endorhizosphere (within root), rhizoplane (root surface) and ectorhizosphere (closely associated soil) with a diverse range of microbes inhabiting each (Figure 1.5) (Hirsch and Mauchline, 2012). The rhizosphere contains more available carbon than the surrounding bulk soil (soil which is not directly associated with plant roots) and supports a higher level of microbial biomass (Fierer, 2017). This available carbon comes from carbon and nitrogen rich compounds which are released from root tissues including; mono- and polysaccharides, phenolic compounds, organic acids, proteins, amino acids and plant cells (Tian et al., 2020). These 'rhizodeposits' appear in different forms including sloughed-off root cells, plant exudates, lysates, mucilage and volatile organic compounds (Tian et al., 2020). Rhizodeposits are thought to play a key role in moulding rhizosphere microbiomes with evidence indicating plants can actively alter rhizodeposition to shape their resident rhizosphere microbiome (Durán et al., 2018, Edwards et al., 2015, Tian et al., 2020). It has also recently been shown that the microbiota itself can modulate the exudation of plant derived metabolites in the rhizosphere, illustrating the complex and intertwined nature of the rhizosphere microbiome and the plant host (Korenblum et al., 2020).

The assembly of the rhizosphere microbiome is known to be modulated by a wide range of abiotic and biotic factors in addition to and in conjunction with rhizodeposition. A recent review of abiotic factors affecting rhizosphere microbiomes pointed to a number of studies in which soil structure, water content, mineral content, pH, temperature, organic matter and agricultural practices were found to influence rhizosphere microbiome assembly (Gustavo Santoyo, 2017). Biotic factors such as the particular plant species and genotype (Micallef et al., 2009, Yu and Hochholdinger, 2018), plant developmental stage (Chaparro et al., 2014, Micallef et al., 2009), expression of immune phytohormones (Lebeis et al., 2015, Yu et al., 2019) and microbe – microbe interactions (Lakshmanan et al., 2014) can influence rhizosphere

microbiome assembly. It is important to note that these factors do not exist and act individually but in the context of all the abiotic and biotic factors present in the rhizosphere. This complexity is noted throughout investigations of rhizosphere microbiomes with differing combinations of the aforementioned factors influencing assembly in specific situations. Therefore, it is also important to note that our understanding of the rhizosphere microbiome is constantly evolving with new findings and advancements in ecological theory advancing rhizosphere microbiome research.

1.2.2 Rhizosphere microbiomes and plant health

The microbial constituents of the rhizosphere functionally interact with the plant in a myriad of ways. Some act as symbionts which promote plant growth, others are parasites, being the causative agents of plant diseases and others are neutral commensals **(Figure 1.6)** (Berendsen et al., 2012). The importance of the rhizosphere

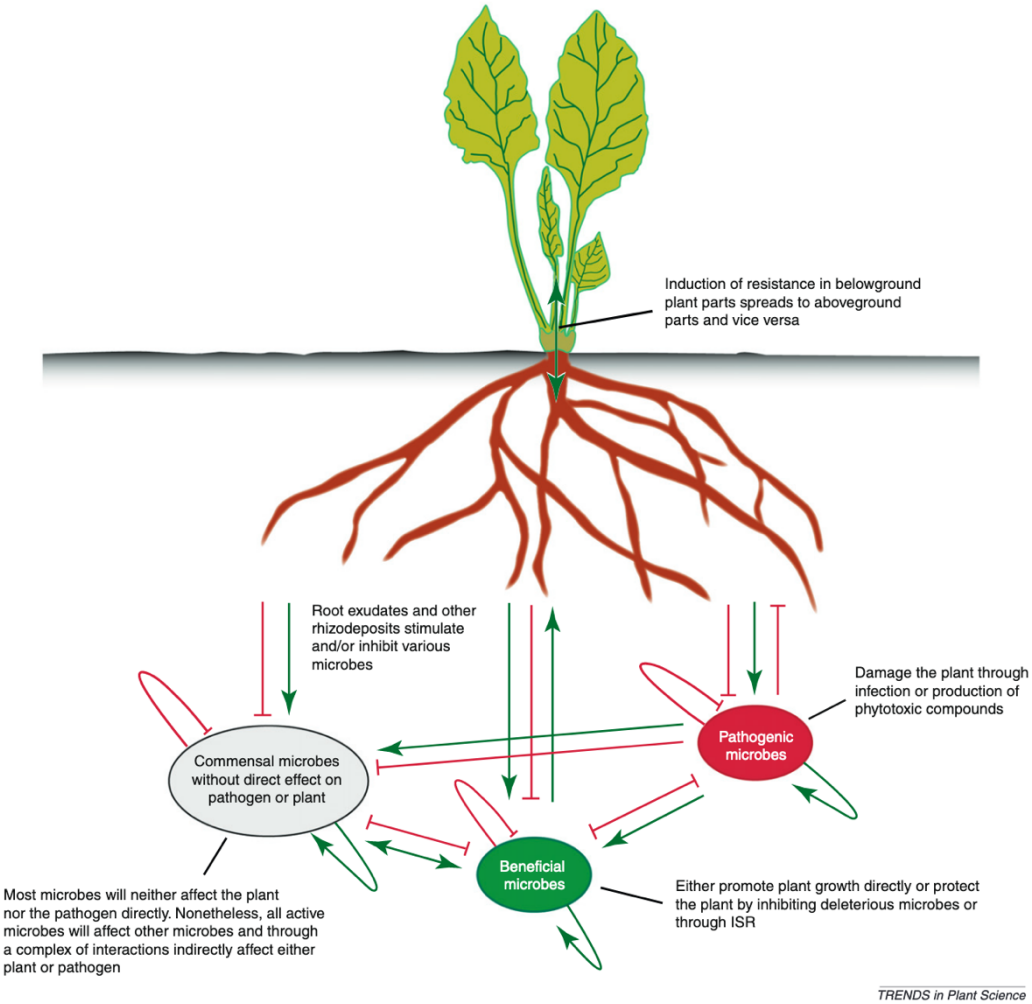

Figure 1.7 Diagram detailing the various interactions and roles of the rhizosphere microbiome in relation to the plant root. *Taken from (Berendsen et al., 2012).*

microbiome to plant health has lead it to be referred to as a plants 'second genome' (Berendsen et al., 2012). Current thinking frames the rhizosphere microbiome in terms of the 'Holobiont Theory' which views host associated microbiota as central to host biology, ecology, and evolution (Hassani et al., 2018, Simon et al., 2019).

As previously described in section 1.1.2, rhizosphere associated microbiota can be causative agents of serious plant diseases and can represent a threat to plant survival. However, beneficial interactions between the plant host and rhizosphere microbiota also occur. Beneficial bacteria and fungi can associate with plants as endophytes (within root), epiphytes (root surface) and the fungi as mycorrhizas, resulting in mutually beneficial symbioses (Chen et al., 2018, White et al., 2019). Endophytic and epiphytic bacteria and fungi can confer beneficial traits to the plant host including resistance to diseases and abiotic stress factors, nutrient acquisition, and protection from herbivory (Gaiero et al., 2013, Lau and Lennon, 2012, Sarangthem Indira Devi, 2015). Arbuscular mycorrhizas (AM) are particularly important, being the most common microbial symbiosis. During the development of this symbiosis, plant cell reprogramming occurs in response to AM colonisation and specific symbiotic tissues known as arbuscules form (Chen et al., 2018). AM fungi can have various beneficial effects on plant health including nutrient acquisition and protection from pathogenic taxa. Subsequently, AM fungi are popular microbial inoculants for boosting plant health and growth in many plant species (Chen et al., 2018).

Microbial assembly at the community level has also recently been implicated in boosting crop biomass and yield, indicating that community level dynamics are important to plant health. An analysis of 'ultra-high' yield rice paddies in Taoyuan China, found that major abiotic and edaphic factors only explained ~ 60% of the boosted rice yield, with the associated microbiomes (which were compositionally and structurally distinct compared to those of comparative sites without boosted yields) a major contributor to 'ultra-high' yields (Zhong et al., 2020). Therefore, understanding and harnessing microbial interactions in the rhizosphere is particularly important for improving plant health and fitness and for the development of crop improvement strategies.

1.2.3 Harnessing rhizosphere microbiomes for crop improvement strategies

As discussed in the preceding section (1.2.3), microorganisms which inhabit the root and rhizosphere soil of plants can confer many beneficial traits upon their plant host (Berendsen et al., 2012, Gaiero et al., 2013, Lau and Lennon, 2012, Sarangthem Indira Devi, 2015). In the context of agricultural systems, this conference of beneficial traits can have important implications for crop security and final yields. Indeed, investigating beneficial microorganisms is of great interest for boosting yields through improvement of pathogen suppression and nutrient acquisition (Gopal and Gupta, 2016, Toju et al., 2018). Many studies have focused on the investigation of individual microbial isolates for use as biocontrol agents and plant growth promoters across a range of major crop species including, *Oryza sativa*, *Triticum aestivum* and *Glycine max* (Martins et al., 2018, Spence et al., 2014, Wang et al., 2020). AM fungi in particular have become widely available commercial bioinoculants for use in agriculture and horticulture (Chen et al., 2018). However, the usage of plant associated microbes to these ends is not restricted to employing a single isolate but can be achieved through using defined microbial communities. For example, a field experiment in which strawberry beds were treated with a defined community showed the treatment being able to alter the microbial community associated with the plants and was associated with enhanced root growth and nitrogen/carbon assimilation in leaves (Kutschera and Khanna, 2016).

There is also increasing interest in moving beyond bioinoculant strategies, by engineering whole rhizosphere microbiomes *in situ* using the plant host themselves (Foo et al., 2017, Jochum et al., 2019, Trivedi et al., 2017, Quiza et al., 2015). If possible, crop beneficial microbiomes could be developed and deleterious microbiomes supressed through rotation strategies with minimal intervention. Host mediated microbiome engineering relies on the genetic and phenotypic traits of the plant host (such as the biotic factors described in section 1.2.1) to influence the assembly of associated microbiomes. Indeed crop genotype (or cultivar) has been shown to influence the root and rhizosphere microbiome in a variety of crop families including; the Poaceae (Sorghum (Schlemper et al., 2017)), the Rosaceae (Strawberry (Nallanchakravarthula et al., 2014)) and the Brassicaceae (*Boechera stricta* (Wagner et al., 2016) and *Arabidopsis thaliana* (Micallef et al., 2009)). In opposition, genotype has also been shown to have no influence on the root and rhizosphere soil microbiome

in other crops, with soil characteristics identified as being more influential, as is the case for the Poaceae family member, Maize (Chen et al., 2017). In addition, more specific factors such as plant developmental stage can work in concert with genotype to influence associated microbiomes (Na et al., 2019).This suggests that the influence of genotype may be situational and/or crop specific. However, this strategy represents a promising tool for beneficial microbiome engineering and merits further research to unpack the relationship between host genetics and microbial assembly.

1.2.4 Molecular methods for analysing rhizosphere microbiome assembly

Before the development of molecular based methods, the microbiological profile of an environment was assessed using culture-based methods. However, it is estimated that less than 1 % of microorganisms can be successfully cultivated with current culture based techniques (Epstein, 2013). The development of molecular based methods enabled the simultaneous identification and relative quantification of culturable and un-culturable microorganisms in environmental samples. In addition, gene and protein expression profiling techniques have enabled the analysis of functionality of microbiomes. These molecular technique advancements have revolutionised microbiome research and our ability to document and analyse microbial ecosystems including soil and rhizosphere microbiomes. Indeed, most investigations discussed in the preceding sections (1.2.1, 1.2.2 & 1.2.3) utilise molecular based methods to analyse rhizosphere microbiome assembly and diversity, particularly amplicon sequencing.

1.2.4.1 Amplicon sequencing

Amplicon sequencing is one of the most common molecular method of analysing soil and rhizosphere microbiome taxonomic composition and relative abundances (Joos et al., 2020). Indeed, many investigations of OSR root and rhizosphere microbiome assemblies utilise this method (**Table 1.1**). This method targets highly conserved taxonomic marker genes such as the 16S rRNA gene found in bacteria and 18S rRNA gene found in eukaryotes as a method of distinguishing taxonomy (Woese, 1987). The combined nature of highly conserved regions and variable regions within the rRNA gene region aids molecular analysis with conserved regions providing areas for amplification primers to bind and variable regions

allowing the identification of taxonomic divergence (Van de Peer et al., 1996). A variety of other genetic sequences can also be used for taxonomic reference including the ITS regions of fungal 18S rRNA genes and even functional genes (Herbold et al., 2015). This is a key advantage of amplicon sequencing in that it can be extremely specific in targeting single groups of microorganisms. PCR amplification of these reference/functional genes are carried out to create amplicon libraries. Sequencing of these libraries using high throughput next generation sequencing (NGS) platforms such as Illumina® MiSeq™ can generate gigabytes of sequencing data and millions of reads for many samples (Caporaso et al., 2011, Caporaso et al., 2012, Herbold et al., 2015, Kozich et al., 2013).

These reads must subsequently be processed to assign taxonomic identities and provide useable data for downstream ecological analyses. A number of pipelines exist for processing NGS amplicon sequencing data including QIIME (Bolyen et al., 2019, Caporaso et al., 2010) and MOTHUR (Schloss et al., 2009, Schloss and McBain, 2020). Traditionally, sequences have been binned into Operational Taxonomic Units (OTUs) based on sequence similarities, typically 97 %, with a representative sequence (to which taxonomy is assigned) selected to represent all sequences clustered into a single I (Nguyen et al., 2016a, Westcott and Schloss, 2015). This clustering can consolidate sequences with variations due to sequencing and PCR induced errors. However, taxonomically different sequences may be clustered into a single I masking true variation (Joos et al., 2020). Indeed in certain bacterial species differences reference gene sequences are seen at a 99 % similarity level (Nguyen et al., 2016a). Recently, Assigned Sequence Variants (ASVs) have been proposed as an alternative to OTUs. To create ASVs error models are applied to identify sequencing errors whilst also accounting for abundance and sequence similarity i.e. removing sequencing errors whilst recognising taxonomic diversity. This increases the taxonomic resolution of ASVs compared to OTUs (Callahan et al., 2017). However, this method may also mask true taxonomic diversity by identifying diversity as sequencing error (Nguyen et al., 2016a). Regardless of whether OTUs or ASVs are employed, community alpha and beta diversity indices, relative abundances, co-occurrence patterns and correlations to metadata can be analysed and visualised using amplicon sequencing data. This provides insight into community structure and diversity. Examples of publications which do so for OSR root and rhizosphere microbiomes can be seen in

(**Table 1.1**). In addition, inference of taxa function based on OTU/ASV taxonomy can be achieved using PICRUST for 16S rRNA data (Langille et al., 2013) and FunGuild for ITS data (Nguyen et al., 2016b).

Despite its wide use in environmental microbiome research and its apparent advantages, amplicon sequencing is not without limitations. In their comparison of amplicon and shotgun metagenome sequencing, Sharpton (2014), discusses research which points to the main limitations of the amplicon sequencing approach. Firstly, as with any method which utilises the process of PCR amplification, biases can be introduced due to primer selection with the potential impact of underestimating community diversity (Hong et al., 2009). Secondly, it can produce estimates of community diversity which are highly variable, due to sequencing errors and the variation in the power of different gene loci in resolving taxonomy (Jumpstart Consortium Human Microbiome Project Data Generation Working, 2012, Liu et al., 1997, Liu et al., 2008, Schloss, 2010, Wylie et al., 2012). Finally, although function of resident taxa can be inferred from the 16S rRNA gene for bacteria and the ITS region for fungi, there is no direct observance of functional genes which are present in the whole microbiome genome, limiting functional analysis (Langille et al., 2013, Nguyen et al., 2016b, Sharpton, 2014). Despite these limitations, the advantages of amplicon sequencing including the ability to profile specific microbiome members based on taxonomic references genes and the ability to generate large volumes of data for many samples in single NGS run makes amplicon sequencing a popular choice for documenting and analysing microbiomes.

1.2.4.2 Alternative molecular methods for analysing microbial communities

In addition to targeted amplicon sequencing of taxonomic reference genes, there are several other molecular based methods for analysing microbial community assembly and function. These include other amplicon-based methods, whole genome sequencing and 'multi-omics' technologies including, transcriptomics, metabolomics, and proteomics. All these techniques have been employed in the analysis of soil and rhizosphere microbiomes and provide different insights into microbial community assembly and function, with a range of advantages and disadvantages to each technique (Baldrian, 2019).

Denaturing gradient gel electrophoresis (DGGE) (Green et al., 2010, Muyzer et al., 1993) and terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997) analysis, like amplicon sequencing, rely on the amplification of taxonomic reference genes such as the 16S rRNA gene. These methods were developed and utilised before the widespread availability of amplicon sequencing and only provide a community 'fingerprint' based on differential electrophoresis banding patterns. These banding patterns are the result of different denaturation of amplicons DGGE) or restriction enzyme digested amplicon fragment lengths (T-RFLP) based on amplicon sequences (Green et al., 2010, Liu et al., 1997, Muyzer et al., 1993). This allows generation of OTUs or 'Ribotypes', however, clone library sanger sequencing or high throughput amplicon sequencing is required in addition to assign I taxonomy directly (Lindström et al., 2018, Lucas et al., 2018). Other disadvantages of using DGGE and T-RFLP compared to high throughput amplicon sequencing include; an inability to sequence poorly resolved bands (DGGE), an inability to detect populations less than 1 % of community relative abundance (DGGE), poor resolution of complex communities (T-RFLP) and incomplete or non-specific digestion which leads to overestimation of community diversity (T-RFLP) (Avis et al., 2006, Morgan et al., 2017). These disadvantages are particularly notable in the case of soil and rhizosphere microbiomes given their highly diverse nature and the increasing recognition of the importance of 'rare' members of microbiomes to microbiome structure and function (Fierer, 2017, Jousset et al., 2017, Ramirez et al., 2018, Sogin et al., 2006). However, the advantages of these techniques include their quick processing times and affordability compared to amplicon sequencing and other 'omic' analysis methods discussed below.

An alternative approach to the analysis of PCR amplified taxonomic reference genes is metagenome shotgun sequencing. In this technique, sequencing of the total DNA content of environmental samples is carried out (Lucaciu et al., 2019, Sharpton, 2014). This provides key advantages over amplicon sequencing. As analysis is not restricted to sequencing one genome locus this enables functional characterization of reads, providing a more accurate assessment of functionality compared to inference based on 16S rRNA gene or ITS sequences alone (Langille et al., 2013, Nguyen et al., 2016b, Sharpton, 2014). This gives the chance to gain information on who is in the microbiome and what they may be doing from the same analysis. For example, a recent

investigation of blueberry rhizosphere microbiomes identified rhizosphere specific microbiome metabolic functions which may provide the rhizosphere microbiome flexibility in responding to plant host induces stresses and changes (Yurgel et al., 2019). In addition, it is possible to construct full assembled microbial genomes from the metagenome. This offers the potential to assign species and strain level taxonomies to microbial constituents and the exploration of yet known microbial life (Rausch et al., 2019, Sharpton, 2014).

Despite these advantages, there are several challenges presented by this technique. Sharpton (2014) discusses a range of research which identifies these challenges. Metagenome data is inherently complex and large which presents problems in its processing and analysis. Challenges include host DNA overwhelming microbiome DNA in samples (i.e., plant DNA in root and rhizosphere soil samples), incomplete genome coverages for each microbial constituent, lack of overlapping reads for the same gene and difficulty in distinguishing whether overlapping reads belong to the same or different genomes. In addition, shotgun metagenomics tends to be more expensive and computationally demanding compared to amplicon sequencing. Therefore, consideration of the information you want from your investigation and the number of samples to be analysed are key in deciding whether amplicon sequencing or shotgun metagenomics is the appropriate technique.

In comparison to analysis methods discussed above, which are concerned with microbial community DNA sequences, metatranscriptomics is concerned with the analysis of RNA profiles and metaproteomics the analysis of proteins in environmental samples (Lucaciu et al., 2019, Shakya et al., 2019). Both techniques have been employed in the analysis of root and/or rhizosphere soil microbiomes (Chaparro et al., 2014, Lin et al., 2013, Lucaciu et al., 2019) and provide evidence of which genes are being actively expressed (metatranscriptomics) and which proteins are being actively produced (metaproteomics) in the microbiome environment.

Advantages of utilising metatranscriptomics over other omics methods include the ability to analyse the active members of microbiomes through identifying differentially expressed genes and how they form biologically relevant pathways (Chaparro et al., 2014, Lucaciu et al., 2019, Shakya et al., 2019). This provides a key advantage over analysing functionality through shotgun metagenomics which is based on sequencing DNA rather than RNA. However, there are limitations in assigning

taxonomy to transcripts, especially for eukaryotic microorganisms, limiting this techniques ability to fully document the taxonomic diversity of microbiome samples (Lucaciu et al., 2019). In addition, the cost of this technique is higher than that of amplicon sequencing, however, it is noted that these costs are reducing with the development of this technique (Shakya et al., 2019).

Regarding proteomics, there are a number of advantages to its use in environmental microbiome research compared with the other 'omics' methods which analyse microbial DNA and RNA. These include the provision of direct evidence of; proteins in samples, protein interactions, post translational modifications and metabolic function in the microbiome (Lucaciu et al., 2019). However, there are challenges to the use of this technique in rhizosphere microbiome analysis specifically including the low expression of microbial proteins in these samples and the limited scope of information in reference databases used for proteomic analysis (Lucaciu et al., 2019).

Together, amplicon sequencing, shotgun metagenomics, DDGE/T-RFLP, metatranscriptomics and metaproteomics all have advantages and disadvantages concerning the analysis of environmental microbiome samples including soil and rhizosphere microbiomes. Each method provides a different 'view' of the microbiome including structure and diversity, the constituent genomes, and active metabolic pathways and processes. Therefore, consideration of what information is required from a specific investigation, the number of samples, computational ability of resources and cost all factor into the decision of which analysis methods to employ.

1.3 The rhizosphere microbiome of OSR

1.3.1 Current understanding of the OSR rhizosphere microbiomes

Compared to its well-studied and characterised relative *A. thaliana*, the rhizosphere microbiome of OSR has not been extensively investigated despite OSR's agricultural and economic importance and, the importance of soilborne pathogens to OSR health and yields (section 1.1.2). Within the past decade, a handful of studies using current microbiome amplicon sequencing methods have been carried out, with the majority focusing on bacterial and/or fungal taxa (**Table 1.1**). However, a recent study of OSR rhizosphere microbiome assembly and its relationship to yield decline

at the landscape level has been presented, in which bacteria, fungi and protists were analysed (Hilton et al., 2021). This represents the largest study of OSR related microbiomes to date in terms of sample site number, landscape distribution of sites and the relationships between agricultural outcomes and OSR microbiomes.

In general, the studies discussed here (**Table 1.1**) provide insights into; common taxa associated with OSR (especially bacteria), in certain cases 'active' taxa (Gkarmiria et al., 2017) and taxa significantly correlated with OSR yield (Hilton et al., 2021). These studies represent a valuable resource for our current understanding of OSR rhizosphere microbiomes. However, they are not exhaustive and further research into microbiome assembly and functionality is required to fully understand the relationships between the plant host and its resident rhizosphere microbiome.

Table 1.1 Current literature regarding the root and rhizosphere microbiomes of OSR

Reference	Study type	Microbial kingdom(s) assessed	Community analysis method(s)	Study location/ inoculum source
(Hilton et al., 2021)	Field	Bacteria, Fungi, Protist	Nextgen sequencing (MiSeq™) Target region: 16S/18S rRNA / ITS	UK wide OSR cultivation region
(Taye et al., 2020)	Field	Bacteria	Nextgen sequencing (MiSeq™) Target region: 16S rRNA	Saskatchewan, Canada
(Floc'h et al., 2020)	Field	Fungi	Nextgen sequencing (MiSeq™) Target region: ITS	Alberta & Saskatchewan, Canada
(Bazghaleh et al., 2020)	Field	Fungi	Nextgen sequencing (MiSeq™) Target region: ITS	Saskatchewan, Canada
(Schlatter et al., 2019)	Field	Bacteria & Fungi	Nextgen Sequencing (MiSeq™) Target Region: 16S rRNA/ ITS	Washington State, USA
(Lay et al., 2018a)	Field	Bacteria & Fungi	Nextgen Sequencing (MiSeq™) Target Region: 16S rRNA/ ITS	Alberta & Manitoba, Canada
(Hale, 2017)	Field	Bacteria & Fungi	Nextgen Sequencing (MiSeq™) Target Region: 16S rRNA, ITS & 18S rRNA	Hertfordshire, UK

(Zhao et al., 2017)	Field	Bacteria, Fungi & *P. brassicae* (Protist)	qPCR & Nextgen Sequencing (HiSeq™) Target Regions: 16S rRNA/ ITS	Hubei, China
(Rathore et al., 2017)	Field	Bacteria	Nextgen Sequencing (MiSeq™) Target Region: 16S rRNA	Carlow, ROI
(Gkarmiria et al., 2017)	Laboratory Microcosm	Bacteria & Fungi	DNA SIP & 454 Pyrosequencing Target Region: 16S rRNA/ ITS	Ultuna, Sweden
(Hilton et al., 2013)	Field & Laboratory microcosm	Bacteria & Fungi	RT-PCR & TRLFP 16S rRNA/ ITS	Warwickshire, UK

1.3.1.1 Bacteria

Bacterial taxa are by far the most studied kingdom of microbial life in the OSR rhizosphere microbiome, having been profiled in nine of the eleven studies published to date **(Table 1.1).** A range of bacterial taxa are known to associate with the roots and rhizosphere soil of OSR, with the Proteobacteria and Actinobacteria being found to predominate in both compartments in a number of studies (Hilton et al., 2021, Lay et al., 2018a, Rathore et al., 2017, Schlatter et al., 2019, Taye et al., 2020). Other common taxa identified in these studies included the Planctomycetes, Gemmamonadetes and Bacteroidetes. In addition to these community profiling studies which gives insight into common bacterial taxa associated with OSR rhizosphere microbiomes, an investigation of taxa which consume OSR derived carbon has been carried out (Gkarmiria et al., 2017). Taxa which were found to dominate this 'active' community included the; Streptomyces, Rhizobium and Flavobacterium in the roots whereas, the Rhodoplanes and Sphingomonas were found to dominate active rhizosphere communities (Gkarmiria et al., 2017).

Community profiling investigations have also enabled analysis of how specific factors such as pathogen infection and agricultural practices can influence root and rhizosphere soil bacterial microbiome assembly (Hilton et al., 2021, Rathore et al., 2017, Zhao et al., 2017). Regarding pathogen infection, Zhao et al. (2017) identified significant differences in the assembly of bacterial root communities between symptomatic and asymptomatic OSR infected with the protozoan parasite *P. brassicacae*. Asymptomatic roots were found to have bacterial communities with

higher abundances of taxa associated with biological control and plant growth promoting properties, indicating the key role bacterial community assembly can play in ameliorating the negative effects of pathogenic processes in OSR. Indeed, bacterial taxa isolated from OSR root systems have been cultured and screened for beneficial traits such as pathogen protection, with a number of protective taxa identified (Etesami and Alikhani, 2016). In addition, the abundance of certain bacterial taxa such as *Serratia proteamaculans, Arthrobacter* sp., *Pedobacter* sp. and a *Stenotrophomonas* sp. have been positively correlated with increased yields (Lay et al., 2018a). Interestingly, Lay et al. (2018a) point to a number of studies regarding these taxa in which their occurrence or application on OSR conferred pathogen protection (*Stenotrophomonas* sp. & *Serratia proteamaculans* (Alström, 2001)) and boosted yield (*Arthrobacter* sp. (Kloepper et al., 1988)).

Regarding influences of agricultural practices upon bacterial community assembly, Rathore et al. (2017) showed that crop establishment practices can have a significant effect upon the resulting root bacterial communities i.e., between strip tillage and Plow established OSR. However, as part of the landscape scale analysis presented by Hilton et al. (2021), OSR crop rotation lengths were not identified as having a significant influence on root, rhizosphere soil and bulk soil bacterial community assembly.

In their landscape scale study, Hilton et al. (2021) also presented data on co-occurrence networks. Bacteria – bacteria co-occurrences were found to predominate in the bulk soil, rhizosphere soil and root networks. This was followed by bacteria – fungi co-occurrences in the bulk soil and rhizosphere soil, and bacteria – protist co-occurrences in roots. This was greater than the number of expected connections based on the proportion of bacterial OTUs included for network analysis. In addition, different enrichments of bacterial phyla forming co-occurring relationships were identified between rhizosphere soil and root communities with a limited number of bacterial OTUs forming significant relationships with OSR yield in these networks.

1.3.1.2 Fungi

Fungal taxa are the second-most studied microbial Kingdom in the OSR rhizosphere microbiome, having been profiled in seven of the eleven studies published

to date (**Table 1.1**). Community profiling of OSR fungal microbiomes indicate that the Ascomycota and Basidiomycota dominate, followed by the Zygomycota and Chytridiomycota (Gkarmiria et al., 2017, Hilton et al., 2021, Lay et al., 2018a, Schlatter et al., 2019). These phyla (in order) also represent the most 'active' fungi which assimilate OSR derived carbon in the root and rhizosphere soil (Gkarmiria et al., 2017).

Furthermore, we have information on potential inter-taxon interactions among the fungal rhizosphere communities of OSR (Floc'h et al., 2020, Hilton et al., 2021). In their investigation, Floc'h et al. (2020) constructed co-occurrence networks representative of the fungal rhizosphere communities of continuously cultivated OSR over a three-year period. A decrease in network complexity was observed over the course of the investigation with different hub taxa identified in each network. In addition, a core fungal microbiome was identified which solely consisted of the taxon *O. brassicae*, a taxon linked with yield decline (Hilton et al., 2013, Hilton et al., 2021).

Co-occurrence networks were also constructed by Hilton et al. (2021) in their landscape level investigation. These networks also incorporated bacteria, protists, and meta data (specifically yield and OSR rotation length) and provide information on significantly co-occurring taxa across UK OSR rhizosphere microbiomes. From these 'inter-taxon' networks Hilton et al. (2021), identified two fungal taxa of particular interest namely, *O. brassicae* and *Tetracladium maxilleforme*. As noted above, high *O. brassicae* abundance was significantly correlated to lower yields and shorter rotations in by Hilton et al. (2021) and in previous investigations of OSR yield decline (Hilton et al., 2013). In addition, *O. brassicae* was identified as being abundant and widespread across UK OSR. *T. maxilleforme* was of particular interest due to its widespread identification across UK OSR rhizosphere microbiomes and its abundance being significantly correlated to higher yields and longer rotations. *T. maxilleforme* is known as an aquatic hyphomycete, however, it has also been documented as an endophytic species (Selosse et al., 2008). Relatives of the *Helotiales* order to which *T. maxilleforme* belongs, are known to be beneficial symbionts in other plant species (Almario et al., 2017). In addition to these two taxa of interest, Hilton et al. (2021) also noted several general plant pathogenic fungi to be widespread and abundant in UK OSR root and rhizosphere microbiomes.

Together, the research discussed here highlights the potentially pivotal role of root and rhizosphere soil fungi to OSR health and productivity. The developing understanding of the OSR fungal rhizosphere microbiome being potentially pivotal to OSR health and agricultural outcomes warrants the further investigation of the resident fungi in this non-mycorrhizal plant species (Cosme et al., 2018).

1.3.1.3 Protists

Very little is known about the protists in OSR from a community perspective. Apart from the community assembly presented by Hilton et al. (2021), research has mainly focused on investigating individual protist taxa such as the pathogen *P. brassicae*. This has included study of *P. brassicae* taxonomic diversity (Bass et al., 2018) and as previously discussed in section 1.3.1.1, the effects of *P. brassicae* infection upon plant growth and bacterial endosphere community assembly (Zhao et al., 2017). In their assessment of protists community assembly, Hilton et al. (2021) found that the SAR supergroup (Stramenopiles, Alveolata and Rhizaria) dominated bulk soil, rhizosphere soil and root protist microbiomes. In the same study, agricultural practices such as the rotation frequency of OSR was not found to significantly influence the assembly of bulk soil, rhizosphere soil and root protist microbiomes. However, Hilton et al. (2021) did identify specific protist taxa from inter-taxon co-occurrence networks which were correlated with shorter rotations and lower yields. Of particular interest was the most highly connected protist OTU P14 (related to the Olpidiopsidales and Haliphtorales clades) which was found to significantly co-occur with the pathogenic fungal taxa *P. brassicae* and *O. brassicae*, and significantly co-exclude with the potentially beneficial endophyte *T. maxilleforme*. This indicates that protist taxa may also influence plant health through indirect means and help shape the assemblies of other microbial kingdoms.

Together the research discussed here indicates that protists may play important roles in the shaping of OSR rhizosphere microbiomes with impacts on plant health and yield. However, the small body of research concerning OSR protist microbiome assemblies and the focus of functional analysis on individual pathogenic taxa limits our understanding of protist in the rhizosphere and warrants further investigation. This is especially true in the light of research which points to key functions protists play in

the rhizosphere such as nutrient cycling, predation of microorganisms, increasing beneficial plant hormone concentrations and disease suppression (Gao et al., 2019, Xiong et al., 2020).

1.3.2 Key knowledge gaps

Despite the apparent importance of many soilborne microbial taxa to OSR (as identified in section 1.1.2), a systemic understanding of how the soil microbiota assembles and functions in relation to OSR is not fully developed. Recent research such as the landscape level analysis of rhizosphere microbiomes presented by Hilton et al. (2021) have made strides towards this goal and provide invaluable insight into the assemblies of bacteria, fungi and protists associated with OSR. It also provides insight into how these microbial kingdoms may interact with each other and how they correlate with agricultural practices and outcomes such as rotation length and final yields. However, several key knowledge gaps still exist in our understanding of OSR rhizosphere microbiomes and rhizosphere microbiomes in general.

There has been limited investigation into how factors such as plant genetics influence microbiome assembly in OSR and plants in general. This is particularly pressing given the increased interest in using the host plant genetics and biological processes (i.e., phenotypically distinct cultivars and the plant immune system) as a means for lasting microbiome manipulation in agricultural systems (section 1.2.3). In addition, functional analysis of the OSR rhizosphere microbiota is sparse beyond investigations of known pathogens (section 1.1.2) and a handful of studies regarding beneficial endophytes (Card et al., 2015, Etesami and Alikhani, 2016). This limits the development of microbial based crop protection strategies in OSR and our general understanding of microbe – plant interactions in this crop species. This is especially true in light of current research which challenges our understanding of plant - microbe interactions in non-mycorrhizal plants (Cosme et al., 2018) and the developing understanding of a number of fungal taxa as key players in OSR yield outcomes (Hilton et al., 2013, Hilton et al., 2021).

Therefore, investigation of these knowledge gaps is of particular interest for OSR and plants in general given the known plant health dynamics of the rhizosphere

microbiota, the contextualisation of the rhizosphere as an essential component of the plant and the interest in rhizosphere microbiome manipulation as an agricultural tool.

1.4 Thesis aims

In Chapter two, it was hypothesised that OSR genotypes with distinct root architecture and plant metabolite exudation profiles could be able to assemble compositionally and structurally different root and rhizosphere soil microbiomes due to their genotypic differences. This enabled examination of plant-genotype microbiome dynamics and informed the ability of using plant genotype as a microbiome manipulation tool. To these ends the molecular characterisation of rhizosphere microbial community assemblies in four distinct OSR genotypes was carried out and enabled determination of whether:

1. Distinct OSR genotypes assemble distinct microbiome assemblies.
2. OSR genotypes differentially recruit microbial taxa which are beneficial or deleterious to plant biomass and final yields.
3. OSR genotypes have distinct inter-taxon relationships using microbial network analysis.

In Chapter three, it was hypothesised that using a combination of cultivation based and sequencing based investigations would help isolate and select ecologically and functionally relevant OSR associated fungi for further investigation .To these ends, a culture collection of OSR rhizosphere associated fungi was isolated and curated using the landscape level OSR microbiome dataset presented by Hilton et al., (2021) to:

1. Isolate a collection of fungal isolates from OSR roots and rhizosphere soil which is representative of the UK OSR rhizosphere mycobiome.

2. Select ecologically relevant isolates which were either; widely distributed, highly abundant and/or significantly correlated with OSR biomass for prioritisation in co-incubation experiments.

3. Carry out co-incubation experiments using isolates of interest on OSR seedlings to screen for functionally beneficial or deleterious fungal isolates.

In Chapter four, it was hypothesised that immune phytohormone mutant and wild-type *A. thaliana* genotypes could be used to elucidate the relationship between fungal root microbiome assembly and plant immune phytohormones and whether 'deleterious' and 'beneficial' root mycobiomes formed. To these ends, the molecular characterisation of root mycobiome community assemblies in three immune phytohormone mutant *A. thaliana* genotypes were carried out to:

1. Determine how root mycobiomes assemble in two immune impaired genotypes of *A. thaliana* deficient in either Abscisic and Salicylic acid biosynthesis, and one *A. thaliana* genotype hypersensitive to Abscisic acid.

2. Determine whether immune deficient genotypes assemble 'deleterious' microbiomes compared to wild-type through examination of functional guilds and the relationship between mycobiome assembly and plant biomass.

3. Determine whether transplantation of wild-type and immune deficient genotype conditioned soils can have a beneficial or compounding legacy effect on subsequent plant biomass and root mycobiome assembly.

2 Rhizosphere microbiome assembly and OSR genotypic diversity

2.1 Introduction

Rhizosphere microbiomes are known as key determinants of plant health (Berendsen et al., 2012). As such, research focusing on harnessing rhizosphere microbiomes to improve crop health and yield, is gaining increasing interest as an agricultural tool (Busby et al., 2017, Foo et al., 2017, Trivedi et al., 2017). However, a systematic understanding of how the microbiome assembles and functions as a whole in relation to the plant host is not fully developed. Indeed, one of the key research priorities for the development of microbiome harnessing strategies is uncovering how the microbiota assemble in response to abiotic and biotic factors which influence the rhizosphere (Busby et al., 2017). A factor of particular interest is host plant genetic variation. This is due to the resulting variation in phenotypic expressions such as, exudate profiles (Hunter et al., 2014), root architecture (Thomas et al., 2016) and biotic and abiotic stress protection (Jochum et al., 2019, Voorrips, 1992). As such, examining the influences of host genotype upon rhizosphere microbiome assembly and function could enable the use of crop genotype (or cultivar) as a method for engineering beneficial microbiomes (Foo et al., 2017).

Crop genotype has been shown to influence the root and rhizosphere soil microbiome in a variety of plant families including; the Poaceae (*Sorghum bicolor* (Schlemper et al., 2017)), the Rosaceae (*Fragaria ananassa* (Nallanchakravarthula et al., 2014)) and the Brassicaceae (*A. thaliana* (Micallef et al., 2009)). However, genotype has also been shown to have no influence on the root and rhizosphere microbiome in other plant species, with soil and environmental characteristics identified as being more influential, as is the case for the Poaceae family member, Maize (Chen et al., 2017) and the Brassicaceae family member, *Boechera stricta* (Wagner et al., 2016). In addition, more specific factors such as the host plants developmental stage has been found to work in concert with genotype to influence microbiome assembly (Na et al., 2019). This suggest that the influence of plant

genotype may rely on the specific environmental context, with a range of factors such as plant development stage and edaphic factors contributing to microbiome formation.

Regarding genotypic variation in OSR, previous investigations have mainly focused on the assessment of plant biological parameters such as exudate profiles (Hunter et al., 2014), agronomic outcomes (Harker et al., 2015) and interactions with specific microorganisms such as the pathogenic Cercozoan protist *P. brassicae* (Mougel et al., 2018). Research on the relationship between OSR genotype and microbiome assembly has mainly focused on bacterial microbiomes using traditional microbiome profiling techniques such as Fatty Acid Methyl Ester (FAME) analysis (Dunfield and Germida, 2001, Dunfield and Germida, 2003, Granér et al., 2003, Siciliano and Germida, 1999) (**Table 2.1**). However, three recent publications using Next Generation sequencing technologies (specifically amplicon sequencing) have assessed the effects of OSR genotype on bacterial (Taye et al., 2020) and fungal (Bazghaleh et al., 2020, Floc'h et al., 2020) root and rhizosphere soil microbiomes (**Table 2.1**).

These studies analysed OSR genotypes with differing levels of commercially significant compounds (erucic acid and glucosinolate) (Taye et al., 2020), herbicide resistance (Floc'h et al., 2020) and geographic origin (Bazghaleh et al., 2020). In their publication Taye et al. (2020) assessed the rhizosphere bacterial microbiomes of 16 canola genotypes in comparison to a reference genotype, with 152 significant differentially abundant bacterial genera being identified in at least one genotype. In addition, a 'core' microbiome (found in all genotypes) was also identified. Regarding genotypic influence upon fungal communities, Bazghaleh et al. (2020) identified variation in the diversity of root and rhizosphere fungal communities between the assessed genotypes. However, no genotype influence was identified by Floc'h et al. (2020) in their study of fungal rhizosphere microbiomes. Together these studies indicate that OSR genotype can influence the assembly of rhizosphere microbiomes dependent on the specific genotype. However, they lack key analyses which are necessary to provide a wholistic understanding of relationship between rhizosphere microbiome assembly and plant host genotypic variation.

Table 2.1 Literature regarding the influence of plant genotype upon the associated microbiomes of OSR.

Reference	Genotypes used in study	Microbial kingdom(s) assessed	Community analysis method	Plant tissue assessed	Genotype effect
(Taye et al., 2020)	16 Genotypes selected based on erucic acid, glucosinolate & fibre content.	Bacteria	Nextgen sequencing (MiSeq™) Target region: 16S rRNA	Rhizosphere	Yes
(Bazghaleh et al., 2020)	17 Genotypes selected based on origin.	Fungi	Nextgen sequencing (MiSeq™) Target region: ITS	Rhizosphere & Root	Yes
(Floc'h et al., 2020)	Roundup Ready® (CRR) & Liberty Link® (CLL)	Fungi	Nextgen sequencing (MiSeq™) Target region: ITS	Rhizosphere	No
(Rochefort et al., 2019)	Astrid, Aviso, Boston, Colvert, Express, Major, Mohican, Tenor & Zorro	Bacteria, Fungi	Nextgen sequencing (MiSeq™) Target region: *gyrB* / ITS	Seed	Yes
(Rybakova et al., 2017)	Avatar, Sherpa & Traviata	Bacteria	Nextgen sequencing (MiSeq™) Target region: 16S rRNA	Seed	Yes
(Dunfield and Germida, 2003)	Excel & Quest	Bacteria	FAME & TRFLP	Rhizosphere & Root	Yes
(Granér et al., 2003)	Express, Libraska, Maluka & Westar	Bacteria (Endophyte)	FAME	Seed, Shoot, Root	Yes
(Dunfield and Germida, 2001)	45A71, Excel, Exceed, Fairview, Hyola, Innovator, Invigor & Quest	Bacteria	FAME	Rhizosphere and Root	Yes
(Siciliano and Germida, 1999)	Excel & Quest	Bacteria (Endophyte)	FAME	Root	Yes

Firstly, the focus on individual microbial kingdoms (**Table 2.1)** precludes the ability to provide a wholistic understanding of microbiome assembly. The omission of protist taxa is particularly concerning given the evolving understanding of key functions protists play in the rhizosphere such as nutrient cycling, predation of microorganisms, increasing beneficial plant hormone concentrations and disease suppression (Gao et al., 2019, Xiong et al., 2020).

The limitation of analyses to individual microbial kingdoms also precludes the ability to investigate potential inter-taxon relationships through examination of inter - taxon co-occurrence networks. Network analysis centres around the study of co-occurrence patterns amongst species in a particular biome ('positive' co-occurring interactions and 'negative' co-excluding interactions). This analysis method is widely employed in the study of macro-ecosystems as a method of analysing community stability and species robustness (Saavedra et al., 2011, Thébault and Fontaine, 2010). It has since become more popular in the study of various microbiomes including; mammalian (Rao et al., 2020), phyllosphere (Pauvert et al., 2019), rhizosphere soil and root microbiomes (Hilton et al., 2021, Rossmann et al., 2020, Zhong et al., 2020), for the identification of keystone taxa and beneficial/deleterious network structures (Banerjee et al., 2018). Investigations of root and rhizosphere microbiomes in agricultural systems have revealed the development of distinct inter-taxon co-occurrence networks between; rhizosphere soil and root compartments (Hilton et al., 2021), average yield and ultra-high yield rice paddies (Zhong et al., 2020), high intensity and organic farmland (Banerjee et al., 2019) and landraces and modern cultivars of wheat (Rossmann et al., 2020). Furthermore, a number of studies were able to identify and replicate *in vivo*, interactions which were; ecologically relevant (Wang et al., 2017) and beneficial to plant health (Durán et al., 2018), using these inferred microbial interaction networks. Therefore, investigating inter-taxon interactions and the networks they form is key to developing our understanding of the OSR rhizosphere microbiomes from an ecological and technological perspective, particularly when investigating the ability of specific OSR genotypes to manipulate rhizosphere microbiomes for beneficial outcomes.

This technique has recently been employed by Hilton et al. (2021) in which bacterial, fungal and protist co-occurrence network members and structures were found to significantly correlate with OSR yield and rotation length. Of note was the

co-occurrence/ co-exclusion relationship between protist OTU P14 and fungal taxa significantly correlated to yields. OTU P14 was found to significantly co-occur with the pathogenic fungal taxa *P. brassicae* and *O. brassicae* (negatively correlated with yield), and significantly co-exclude with the potentially beneficial endophyte *T. maxilleforme* (positively correlated with yield).

Finally, how rhizosphere microbiome assembly correlates with yield is also absent from previous genotype studies, preventing linkage of taxa abundances and inter-taxon co-occurrence networks to yield. Therefore, addressing these key omissions is essential to understand plant genotype – microbiome dynamics and the development of plant genotype as a microbiome manipulation tool.

2.2 Aims

In this chapter, it was hypothesised that OSR genotypes with distinct root architecture and plant metabolite exudation profiles could be able to assemble compositionally and structurally different root and rhizosphere soil microbiomes due to their genotypic differences. This enabled examination of plant-genotype microbiome dynamics and informed the ability of using plant genotype as a microbiome manipulation tool. To these ends the molecular characterisation of rhizosphere microbial community assemblies in four distinct OSR genotypes was carried out and enabled determination of whether:

1. Distinct OSR genotypes assemble distinct microbiome assemblies.
2. OSR genotypes differentially recruit microbial taxa which are beneficial or deleterious to plant biomass and final yields.
3. OSR genotypes have distinct inter-taxon relationships using microbial network analysis.

2.3 Materials and Methods

2.3.1 Experimental area and design

Four genotypes of OSR (*Brassica napus*) representing extremes of plant root exudate profiles and root density were selected for use in this study. Canard DH13 and Temple were selected as representatives of extremes of plant metabolite exudation profiles. Canard DH13 and Temple were found to have significant differences in the exudation concentrations of; Malate, Citrate, Succinate, Sucrose, Choline and Fumarate (**Table 2.2**), with Canard DH13 representative of low and Temple high exudation concentrations. Genotypes selected based on root architecture (Catana and Compass) had a significant difference in root biomass with Catana representative of the low biomass extreme and Compass the high biomass extreme (**Figure 2.1**)(Thomas et al., 2016).

Table 2.2 Mean concentrations (mg g-1 dry root) of the six different compounds identified from root exudate profiling present in the exudates of the Temple and Canard OSR genotypes used in this study. *Data taken from pending publication.*

exudate (mg g^{-1} dry root)	temple	canard	LSD ($p > 0.05$)	fold change
malate	3.58	1.53	0.70	2.3
citrate	1.70	0.96	0.22	1.8
succinate	0.36	0.23	0.05	1.6
sucrose	0.34	0.06	0.07	5.2
choline	0.08	0.04	0.01	1.9
fumarate	0.04	0.01	0.01	4.1

These genotypes were grown at the Cottage Field West field site at the University of Warwick Wellesbourne campus (**Figure 2.A**). The field site consists of a Wick Series sandy loam soil (16.3 % clay, 16.6 % silt and 67.1 % sand) with a carbon content of 0.9 % and pH of 6.8 (Whitfield, 1974). This field has no history of OSR cultivation since establishment of the National Vegetable Research Station (currently University of Warwick Wellesbourne campus) in 1949. The experiment was conducted over one growing season with seeds sown 4th Sept. 2014, microbiome samples taken 20th April 2015 and mature crop harvested 31st July 2015. Genotypes

were sown at 100 seeds/m^2 in specific plots following a randomly allocated design with twenty-one replicate plots for each genotype (**Figure 2.B**). Plots were 6 m x 3 m in dimension with 2 m between plots in an east to west direction and 5 m between plots in a north to south direction. Plots were covered with hoops and netting to prevent bird damage from 11th Sept. 2014 – 15th April 2015. Plots were successively treated with slug pellets and were sprayed with; Metazachlor 1.5 l ha^{-1} (5th Sept. 2014), Hallmark® (Lambda-cyhalothrin) 100 ml ha^{-1} (16th Sept. 2014) and 75 ml ha^{-1} (8th April 2015), Prosaro® (Tebuconazole, Prothioconazole & N,N-Dimethyl decanamide) 0.4 l ha^{-1} (20th Oct. 2014) and 1 l ha^{-1} (20th April 2015), Juventas® (Metoconazole) 0.8 l ha^{-1} (18th March 2015), Nitram® (Ammonium Nitrate) 289.6 kg ha^{-1} (30th March 2015) and Glyphosate 3 l ha^{-1} + Pod-Stick® 1 l ha^{-1} (10th July 2015).

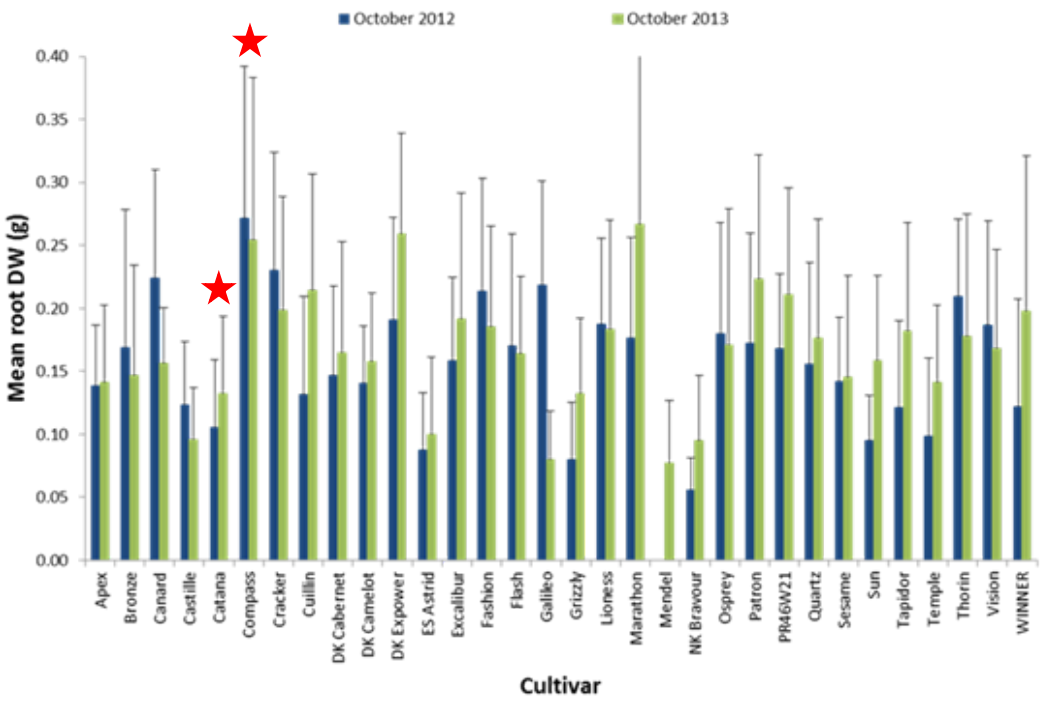

Figure 2.1 Bar chart of dry root biomass of cultivars from Thomas et al.'s (2016) assessment of OSR cultivar root traits. *Figure is adapted from data presented in Thomas et al., (2016). Data is representative of mean dry root weight of OSR plants (+SD) harvested in October 2012 (blue) and October 2013 (green). The cultivars utilised in this study (Catana and Compass) are indicated by red stars.*

A

B

SARISA: Roots of Decline. Year 1 (2014/15) WP3 Trial Design - Cottage Field West

Rows	Cols	1		2		3		4		5		6		7		8		9		10		11		12	
	5m	3m	2m	3m	2m	3m	2m	3m	2m	3m	2m	3m	2m	3m	2m	3m	2m	3m	2m	3m	2m	3m	2m	3m	2m
1	6m	1		2		3		4		29		30		31		32		57		58		59		60	
	5m												N												
2	6m	5		6		7		8		33		34		35		36		61		62		63		64	
	5m										S				E										
3	6m	9		10		11		12		37		38		39		40		65		66		67		68	
	5m												S												
4	6m	13		14		15		16		41		42		43		44		69		70		71		72	
	5m																								
5	6m	17		18		19		20		45		46		47		48		73		74		75		76	
	5m																								
6	6m	21		22		23		24		49		50		51		52		77		78		79		80	
	5m																								
7	6m	25		26		27		28		53		54		55		56		81		82		83		84	
	5m																								

Figure 2.2 (A) Experimental field site (outlined by red box) & (B) experimental design sowing guide. *(**A**) Experimental field site with experimental plots outlined in red. Map obtained from Google Maps™ (**B**) Colours are indicative of genotype sown in each plot (Brown = Compass, Blue = Catana, Green = Temple & Red = Canard.*

2.3.2 Sample collection for microbiome analysis, plant root/shoot weights and final harvest yield data.

Five replicate plants were selected one metre within each plot for collection of rhizosphere soil and root tissues at a depth of 20 cm during the flowering stage of the OSR growth cycle (20th April 2015). Loosely adhering soil was removed from roots by gentle tapping with a 70 % ethanol sterilised trowel, leaving no more than 2 mm rhizosphere soil. Root systems were separated from plants at the root stem interface under sterile conditions in a laminar flow cabinet with rhizosphere soil collected from root systems with two washes in 25 ml of sterile de-ionised water (SDW) in 50 ml Falcon® tubes. Suspended rhizosphere soil was then pelleted in these Flacon® tubes by centrifugation at 30,000 rpm for 15 min with supernatant discarded and pellets stored at -80 ºC for subsequent DNA extraction. Washed root systems were harvested for the collection of lateral roots which were then pooled into a single sample for each plot. From this pooled sample a 0.5 g subsample was taken and frozen at -80 ºC for subsequent DNA extraction. Shoot systems and the remaining root systems were dried in paper bags in an oven at 70 ºC for two days to obtain dry weight data from these plant compartments.

2.3.3 Next Generation amplicon sequencing

2.3.3.1 DNA sample preparation and sequencing

DNA was extracted from 500 µl of rhizosphere soil wash (1g ml^{-1}) or 0.5 g of washed lateral roots using the PowerSoil-htp® 96 well Soil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA). Frozen rhizosphere soil wash and roots were defrosted once for insertion into a PowerSoil-htp® 96 well extraction plate. Extraction plates were kept on dry ice to ensure all samples were not defrosted more than once. Samples from were randomised over different plates to control for plate-to-plate variation. Extracted DNA was quantified with an Invitrogen Qubit® fluorometer 2.0 (Invitrogen brand, Thermo Fisher Scientific, Waltham, USA) using the broad range DNA assay. DNA samples were then diluted to a working concentration between 1 – 10 ng/µl. Analysis of bacterial, fungal and protist communities was carried out using 16S rRNA, ITS rRNA and 18S rRNA gene (respectively) amplicon sequencing. Amplification of the bacterial 16S rRNA v4 region was carried out using primers 515f and 806r (Caporaso et al., 2011). Amplification of the fungal ITS2 rRNA region was carried out using the fITS1 (Ihrmark et al., 2012) and rITS4 (White et al., 1990)

primers. Amplification of the eukaryotic 18S rRNA gene v9 region was carried out for protists using the Euk_1391f and 18S EukBr primers (Amaral-Zettler et al., 2009, Stoeck et al., 2010). These primers were modified at the 5′ end with adapters from a dual-index sequencing strategy (Kozich et al., 2013). In addition to these amplification primers, a blocking primer (Vestheim and Jarman, 2008), was also used to prevent the amplification of OSR sequences in the 18S rRNA gene sequencing PCRs (OSRb 5' GCCCGTCGCTCCTACCGATTGAAGATC 3'). PCR reactions were performed in a reaction volume of 25 µl, containing Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs®, Ipswich, USA) and 0.5 µM of each primer. PCR conditions for 16S , ITS and 18S rRNA genes were as follows; 16S: 95 ºC for 2 min, 30 cycles of 95 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 5 min and then final extension of 72 ºC for 10 min, ITS: 95 ºC for 2 min, 30 cycles of 95 ºC for 30 s, 52 ºC for 30 s, 72 ºC for 2 min and then final extension of 72 ºC for 10 min, and 18S: 95 ºC for 2 min, 30 cycles of 95 ºC for 20 s, 57 ºC for 15 s, 72 ºC for 5 min and then final extension of 72 ºC for 10 min. Amplified DNA samples were then purified using magnetic purification beads. The Illumina® Nextera® Index PCR system (Illumina®, San Diego, USA) was then utilised to tag each samples' sequences for demultiplexing post sequencing. Index PCR reactions were performed in a reaction volume of 25 µl, containing Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs®, Ipswich, USA) and 0.5 µM of each primer. PCR conditions were as follows: 95 °C for 3 min, 8 cycles of 98 °C for 20 s, 55 °C for 15 s, 72 °C for 15 s and 72 °C for 5 min. Following index tagging of sequences, samples were normalised using a SequalPrep™ Normalisation Kit (Invitrogen brand, Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. Sequencing of libraries was carried out using a NextSeq® 550 Midi run (Illumina®, San Diego, USA) at the University of Warwick Genomics facility, Coventry, UK.

2.3.3.2 Post sequencing processing

Raw sequences were provided demultiplexed by the University of Warwick Genomics Facility. Low quality bases from sequence ends were removed using Trimmomatic v0.35 (Bolger et al., 2014). Subsequent steps were performed using USEARCH and UPARSE software (Edgar, 2010, Edgar, 2013). Paired end reads were created for 16S rRNA amplicons by assembling forward and reverse reads with quality filtering using -fastq_maxee 0.5. Single end reads were created using the forward read

for the amplified ITS gene region and amplified 18S rRNA gene due to the variable length of the ITS rRNA region and large size of the 18S rRNA amplicon. Unique sequences were sorted by abundance and singletons were discarded from the dataset using usearch -sortbysize–minsize 2. Sequences were then clustered at a 97 % minimum identity threshold for Operational Taxonomic Units (OTUs) (usearch-cluster_otus) with chimeras removed using the integrated chimera filters. A second chimera filtering step was then carried out using UCHIME (Edgar et al., 2011) and the following taxonomy assignment databases. Taxonomy was assigned using QIIME v1.8 (Caporaso et al., 2010) with the Greengenes reference database (gg_13_8) for 16S rRNA (McDonald et al., 2012), the UNITE database v7.0 for ITS gene region (Kõljalg et al., 2013), and the SILVA (release 119) and PR2 v4.10.0 databases for 18S rRNA (Guillou et al., 2013, Quast et al., 2013). For the resulting 16S rRNA OTU table, mitochondrial and chloroplast 16S rRNA were manually removed, with bacterial reads being retained. Operational Taxonomic Unit (OTU) tables were obtained using MacQiime version 1.9.1 (Caporaso et al., 2010) with chloroplast and mitochondrial reads being removed from the 16S rRNA table. From the 18S rRNA table, sequences from Archaeplastida, fungi and metazoa were removed to leave predominantly single-celled eukaryotes, referred to hereafter as protists. Rarefaction was carried out at 2500, 4000 and 1000 reads for 16S rRNA, ITS gene region and 18S rRNA amplicons (**Figure 2.3**).

2.3.4 Bioinformatic analysis

2.3.4.1 General R software

Bioinformatic analyses were carried out using R software (R Core Team, 2013) with a range of R packages used to analyse OTU tables in the RStudio software environment (RStudio Team, 2015). Unless otherwise stated, statistical analyses were carried out using the R stats package (R Core Team, 2018). Graphs were produced using the ggplot2 package (Wickham, 2016) with the following add-on packages; ggpubr (Kassambara, 2018), ggrepel (Kamil Slowikowski, 2019), ggalt (Bob Rudis, 2017), ggforce (Lin Pedersen, 2019), cowplot (O. Wilke, 2019) and ggfortify (Tang, 2016, Yuan Tang, 2016) unless otherwise stated.

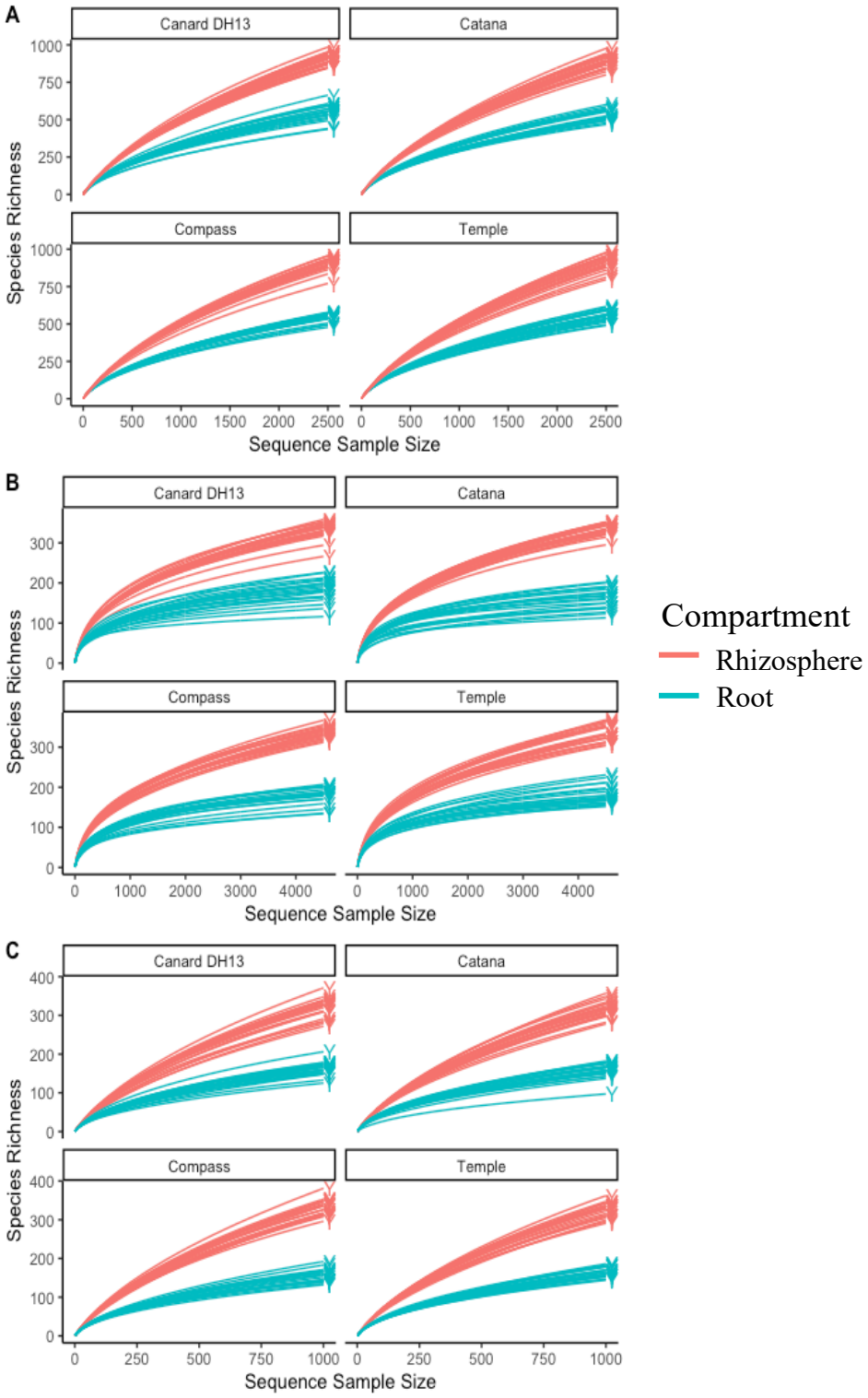

Figure 2.3 Rarefaction curve for Bacteria (A), Fungi (B) and Protist (C) OTU tables. *(A) Bacteria OTU table was rarefied to 2500 reads. (B) Fungi OTU table was rarefied to 4500 reads. (C) Protist OTU table was rarefied to 1000 reads. Plots are faceted by OSR genotype, root and rhizosphere soil samples were indicated by curve colour (blue = root, red = rhizosphere soil). X-axis is representative of number of reads in samples with the y-axis representative of the number of unique species.*

2.3.4.2 Analysis of microbial community sequence data

Rarefied OTU read and relative abundance tables were used to analyse microbial communities in the R software environment using the phyloseq (McMurdie and Holmes, 2013) and vegan (Jari Oksanen 2019) microbial community and microbial ecology analysis packages (unless otherwise stated). Community alpha (α) diversity was determined using the Fisher's Alpha method on the OTU read tables of each amplicon with significance tested for using the Kruskal-Wallis test with a Dunn's Test post-hoc analysis. Community beta (β) diversity was assessed using ANOSIM analysis, which provides a value of community dissimilarity (R^2) and corresponding level of significance (p). R^2 values range from -1 to +1, with positive tending values indicating dissimilarity due to differences between groups and negative tending values indicating a higher level of variation within groups than between groups. Community β-diversity was assessed through cluster analysis based upon Bray Curtis dissimilarity matrices with non-metric multidimensional scaling (NMDS). Community composition at the phylum level was assessed using the Kruskal-Wallis test with a Dunn's Test post-hoc analysis upon relative abundance OTU tables. Community composition at the OTU level was assessed using SIMPER analysis upon relative abundance OTU tables in the PAST3 software (Hammer, 2019). Indicator species analysis was carried out upon rarefied OTU read tables using the indicator analysis within the labdsv R package (Roberts, 2019).

2.3.4.3 Analysis of microbiome influence on OSR biomass

Correlation analysis using the spearman's correlation function in R with the Bonferroni multiple comparison correction was carried out on OTU read tables versus yield and shoot/root weight meta data. This analysis was carried out at every taxonomic level for bacterial, fungal and protist data. Analysis of the effect of overall community structure upon biomass data was carried out using the ADONIS function of the vegan package in R (Jari Oksanen 2019) and visualised using NMDS plots with Ordisurf ordination (Jari Oksanen 2019) of significant metadata.

2.3.4.4 Inter-Taxon co-occurrence network analysis

Merged OTU tables for the creation of inter-taxon co-occurrence networks were generated by taking the upper quartile of the bacterial, fungal and protist relative abundance OTU tables and merging by sample (samples missing from at least one amplicon type were not included in the study, sample numbers as follows; rhizosphere all = 83, root all = 82, Canard (rhizosphere = 21, root = 19) , Catana (rhizosphere = 21, root = 21), Compass (rhizosphere = 19, root = 21), Temple (rhizosphere = 21, root = 19)). Networks were produced using merged OTU tables through FastSpar with 20 iterations and 100 bootstraps (Watts et al., 2019). Networks were subsequently analysed using the Igraph package in R with a correlation threshold of 0.4 (Csardi and Nepusz, 2006). Network statistic plots were generated in R using the within module degree and among module connectivity indices equation described in Olesen et al. (2007), using the node role assignment described in Guimerà and Amaral (2005). Network role alluvial plots were created using the ggplot2 extension ggalluvial (Brunson, 2020). Network chord graphs at the phylum level were produced using the circlize R package (Gu et al., 2014).

2.3.4.5 Statistical analyses

Normality of data assessed using the Shapiro-Wilk normality test where appropriate. Unless otherwise stated in sections 2.3.4.2, 2.3.4.3 and 2.3.4.4, data were assessed using the Kruskal-Wallis test with Dunn's Test post hoc analysis for non-parametric data analysis in R (R Core Team, 2018, R Core Team, 2013).

2.4 Results

2.4.1 Rhizosphere microbiome community diversity

Analysis of microbial community α (Fishers′α) and β-diversity (Bray-Curtis Dissimilarity, NMDS and ANOSIM) identified distinct root and rhizosphere soil microbiomes with a significantly more diverse rhizosphere than root across bacterial, fungal and protist communities (**Figure 2.4**). SIMPER analysis identified many individual OTUs contributing to the dissimilarity of the root and rhizosphere soil microbial communities, with many of the top contributing OTUs being enriched in the root compartment (**Table 2.3**)

Regarding genotype, within the root (**Figure 2.5**) and rhizosphere soil (**Figure 2.6**) compartments distinct microbial communities based on genotype were not identified. However, a low level of dissimilarity in community β diversity was identified between the rhizosphere protist communities of, Canard and Catana and, Canard and Temple (**Figure 2.6**). This dissimilarity was found to be driven by small shifts over many taxa of low relative abundance using SIMPER analysis (**Table 2.4**).

2.4.2 Rhizosphere microbiome community composition

Comparison of taxa relative abundances between root and rhizosphere microbial communities identified significant enrichment of a variety of microbial Phyla in each compartment. In the bacterial microbiomes (**Figure 2.7A**); the Armatimonadetes, Bacteroidetes, Firmicutes and TM7 were significantly enriched in the root whereas; the Actinobacteria, Acidobacteria, Chlorobi, Chloroflexi, Cyanobacteria, Gemmatimonadetes, Verrucomicrobia and WS3 were in the rhizosphere. Regarding fungal communities (**Figure 2.7B**), the Ascomycota dominated both root and rhizosphere communities, however, significant enrichments of certain Ascomycete classes were identified. In the rhizosphere compartment, the Dothideomycetes and Sordariomycetes Classes were significantly enriched whereas the Leotiomycetes Class and Unidentified Ascomycetes were significantly enriched in the root. Similarly, the root and rhizosphere Protist communities were both dominated by the SAR supergroup, with the Alveolata significantly enriched in the root (**Figure 2.7C**).

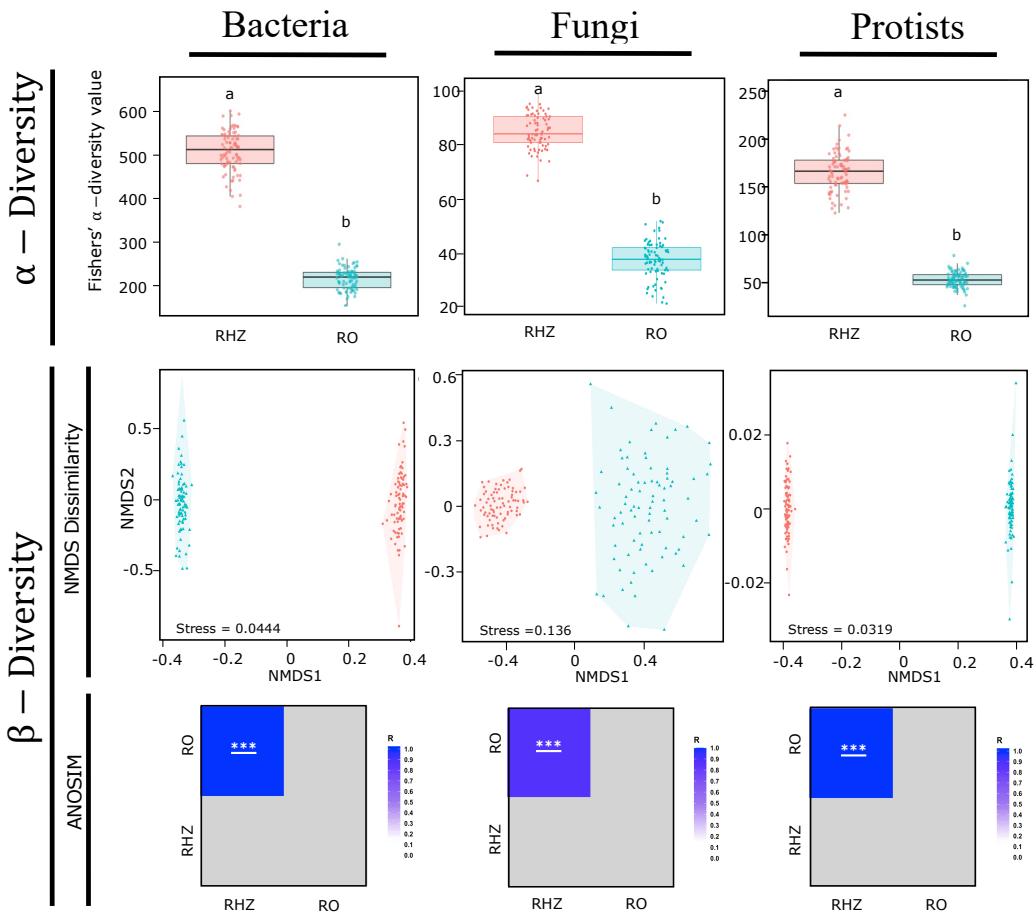

Figure 2.4 Community α & β diversity index analyses for combined root and rhizosphere communities. α diversity analysis: *Box and whisker plots of Fisher's alpha diversity of bacterial, fungal and protist communities in the root and rhizosphere soil microbiomes. Groups with different letters denote significant differences between groups ($p < 0.001$). Statistical analysis was carried out in R using a Kruskal-Wallis test with Dunn's Test post-hoc analysis. N = 84.* ***β diversity analyses (NMDS):*** *Bray-Curtis dissimilarity for bacterial, fungal and protist communities were calculated from rarefied community relative abundance data using the vegan package in R. NMDS scaling was calculated using 1000 bootstraps with the minimal stress noted on the top left-hand corner of plots. Sample compartment is identified by shape and coloration of data points: root (blue triangle) and rhizosphere soil (red circle). Sample clusters based on compartment are denoted using the convex hull. N = 84.* ***β diversity analyses (ANOSIM):*** *ANOSIM analysis for bacterial, fungal and protist communities was calculated suing the vegan package in R. Significant dissimilarities denoted with asterisks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)Significant dissimilarities with $R > 0.2$ are underlined. N = 84.*

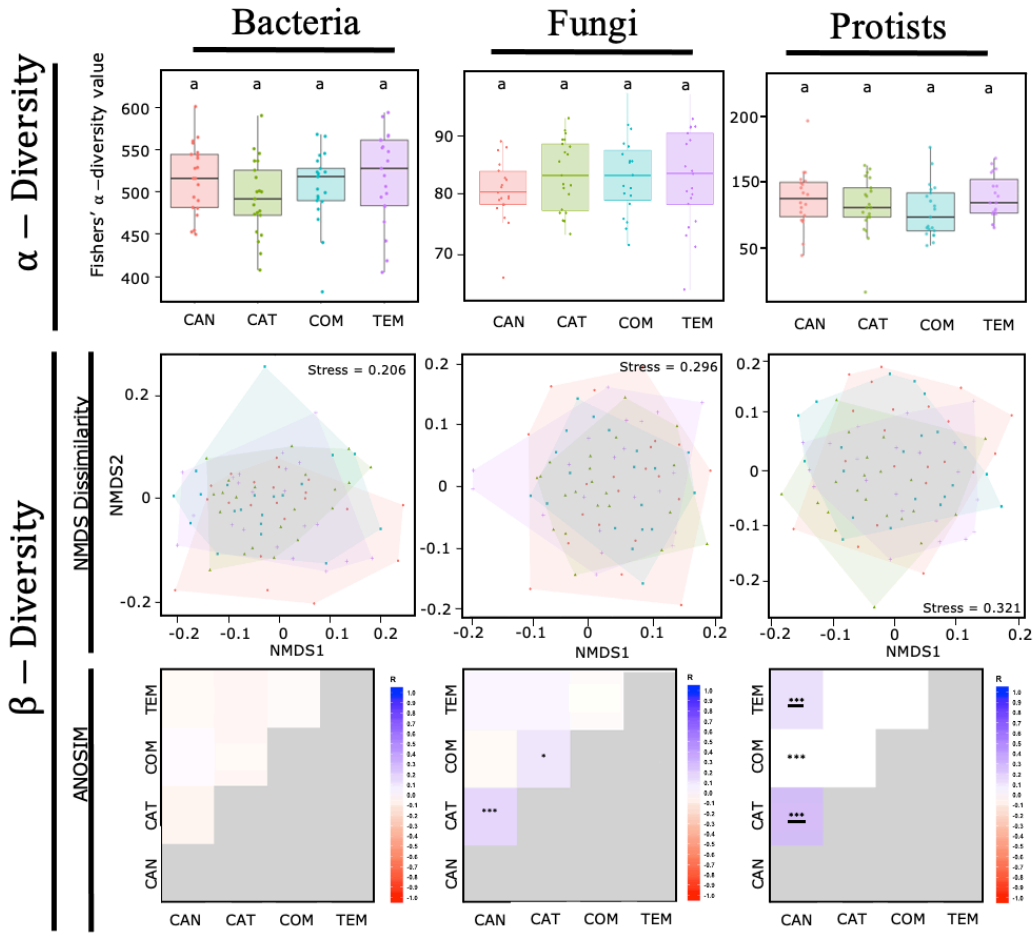

Figure 2.5 Community α & β diversity index analyses for individual genotype rhizosphere soil communities. α diversity analysis: *Box and whisker plots of Fisher's alpha diversity of bacterial, fungal and protist communities in the root and rhizosphere soil microbiomes. Groups with different letters denote significant differences between groups ($p < 0.001$). Statistical analysis was carried out in R using a Kruskal-Wallis test with Dunn's Test post-hoc analysis. N = 21.* ***β diversity analyses (NMDS):*** *Bray-Curtis dissimilarity for bacterial, fungal and protist communities were calculated from rarefied community relative abundance data using the vegan package in R. NMDS scaling was calculated using 1000 bootstraps with the minimal stress noted on the top left-hand corner of plots. Sample genotype (cultivar) is identified by shape and coloration of data points: Canard (red circle), Catana (green triangle), Compass (blue square), Temple (purple cross). Sample clusters based on compartment are denoted using the convex hull. N = 21.* ***β diversity analyses (ANOSIM):*** *ANOSIM analysis for bacterial, fungal and protist communities was calculated suing the vegan package in R. Significant dissimilarities denoted with asterisks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) Significant dissimilarities with $R > 0.2$ are underlined. N = 21*

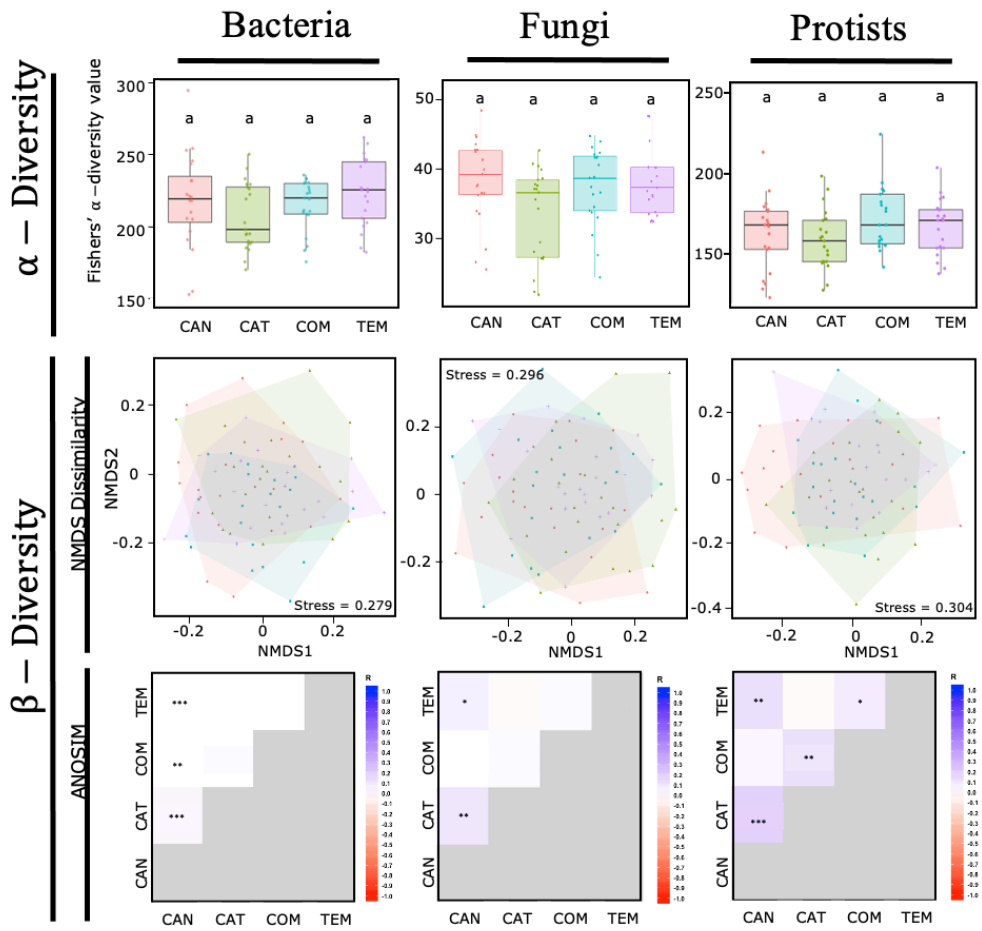

Figure 2.6 Community α & β diversity index analyses for individual genotype root communities. α diversity analysis*: Box and whisker plots of Fisher's alpha diversity of bacterial, fungal and protist communities in the root and rhizosphere soil microbiomes. Groups with different letters denote significant differences between groups ($p < 0.001$). Statistical analysis was carried out in R using a Kruskal-Wallis test with Dunn's Test post-hoc analysis. N = 21.* ***β diversity analyses (NMDS):*** *Bray-Curtis dissimilarity for bacterial, fungal and protist communities were calculated from rarefied community relative abundance data using the vegan package in R. NMDS scaling was calculated using 1000 bootstraps with the minimal stress noted on the top left-hand corner of plots. Sample genotype (cultivar) is identified by shape and coloration of data points: Canard (red circle), Catana (green triangle), Compass (blue square), Temple (purple cross). Sample clusters based on compartment are denoted using the convex hull. N = 21.* ***β diversity analyses (ANOSIM):*** *ANOSIM analysis for bacterial, fungal and protist communities was calculated suing the vegan package in R. Significant dissimilarities denoted with asterisks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) Significant dissimilarities with $R > 0.2$ are underlined. N = 21*

Table 2.3 Top OTUs contributing to dissimilarity between root and rhizosphere soil bacterial, fungal and protist communities as identified by SIMPER analysis.

kingdom	otu	taxonomy	mean dissimilarity	cumulative contribution (%)	mean relative abundance (%)	
				cumulative	rhizosphere soil	root
bacteria	OTU10	(o) Myxococcales	1.11	1.83	0.90	**3.11**
	OTU11	(o) Sphingobacteriales	0.97	3.44	2.62	**4.50**
	OTU17	(o) Pedosphaerales	0.82	4.80	0.97	**2.60**
	OTU6	(o) Sediment-1	0.78	6.10	**1.93**	0.36
	OTU3	(o) Roseiflexales	0.78	7.40	1.45	**2.97**
				cumulative	rhizosphere soil	root
fungi	OTU20	(p) Ascomycota	2.94	4.27	0.45	**6.33**
	OTU10	(o) Helotiales	2.89	8.47	0.84	**6.63**
	OTU4	(c) Sordariomycetes	2.54	12.16	**5.68**	0.68
	OTU6	(k) Fungi	2.15	15.28	3.19	**5.26**
	OTU40	(o) Hymenochaetales	1.93	18.09	1.19	**3.66**
				cumulative	rhizosphere soil	root
protist	OTU8	(g) Uncultured Eustigmatophyceae	5.67	7.72	3.27	**14.60**
	OTU45	(g) Cercomonadida environmental sample	2.37	10.95	0.79	**5.51**
	OTU42	(s) *Nuclearia moebiusi*	2.20	13.94	1.02	**5.42**
	OTU4489	(g) Uncultured Eustigmatophyceae	1.73	16.30	1.94	**5.32**
	OTU72	(g) Uncultured Eimeriidae	1.65	18.54	1.44	**4.72**

Table 2.4 Top OTUs contributing to dissimilarity between rhizosphere Canard, Temple and Catana protist communities as identified by SIMPER analysis.

comparison	taxonomy	mean dissimilarity	cumulative contribution%	mean relative abundance canard (%)	mean relative abundance temple (%)
canard vs temple	OTU31 (g) uncultured eimeriidae	1.67	3.184	**5.26**	2.43
	OTU20 (g) uncultured Oxytrichidae	1.533	6.109	**3.51**	2.16
	OTU47 (g) uncultured stramenopile	1.261	8.513	1.04	**2.74**
	OTU13 (g) uncultured eukaryote	1.095	10.6	4.99	**5.51**
	OTU54 (s) *Euglypha rotunda*	0.8346	12.19	**3.37**	2.96
	OTU25 (s) *Pythium capillosum*	0.7518	13.63	**1.36**	1.18
	OTU35 (g) uncultured Eimeriidae	0.7367	15.03	2	**2.25**
	OTU124 (g) uncultured eukaryote	0.6596	16.29	**1.01**	0.6
	OTU106 (s) *Cyphoderia amphoralis*	0.6251	17.48	**1.38**	1.26
	OTU57 (s) *Euglypha rotunda*	0.6234	18.67	2.57	**2.7**
				mean relative abundance canard (%)	**mean relative abundance catana (%)**
canard vs catana	OTU31 (g) uncultured eimeriidae	1.781	3.382	**5.26**	3.36
	OTU20 (g) uncultured Oxytrichidae	1.665	6.544	**3.51**	1.86
	OTU47 (g) uncultured stramenopile	1.579	9.542	1.04	**3.54**
	OTU13 (g) uncultured eukaryote	1.094	11.62	4.99	**5.74**
	OTU22 (g) uncultured Eustigmatophyceae	0.8697	13.27	0.4	**1.46**

OTU54 (s) *Euglypha rotunda*	0.8431	14.87	**3.37**	3.11
OTU8 (g) uncultured Eustigmatophyceae	0.6938	16.19	2.98	**3.57**
OTU57 *Euglypha rotunda*	0.6296	17.39	2.57	**2.89**
OTU106 *Cyphoderia amphoralis*	0.6229	18.57	**1.38**	1.29
OTU4489 (g) uncultured Eustigmatophyceae	0.5863	19.68	1.27	**2.31**

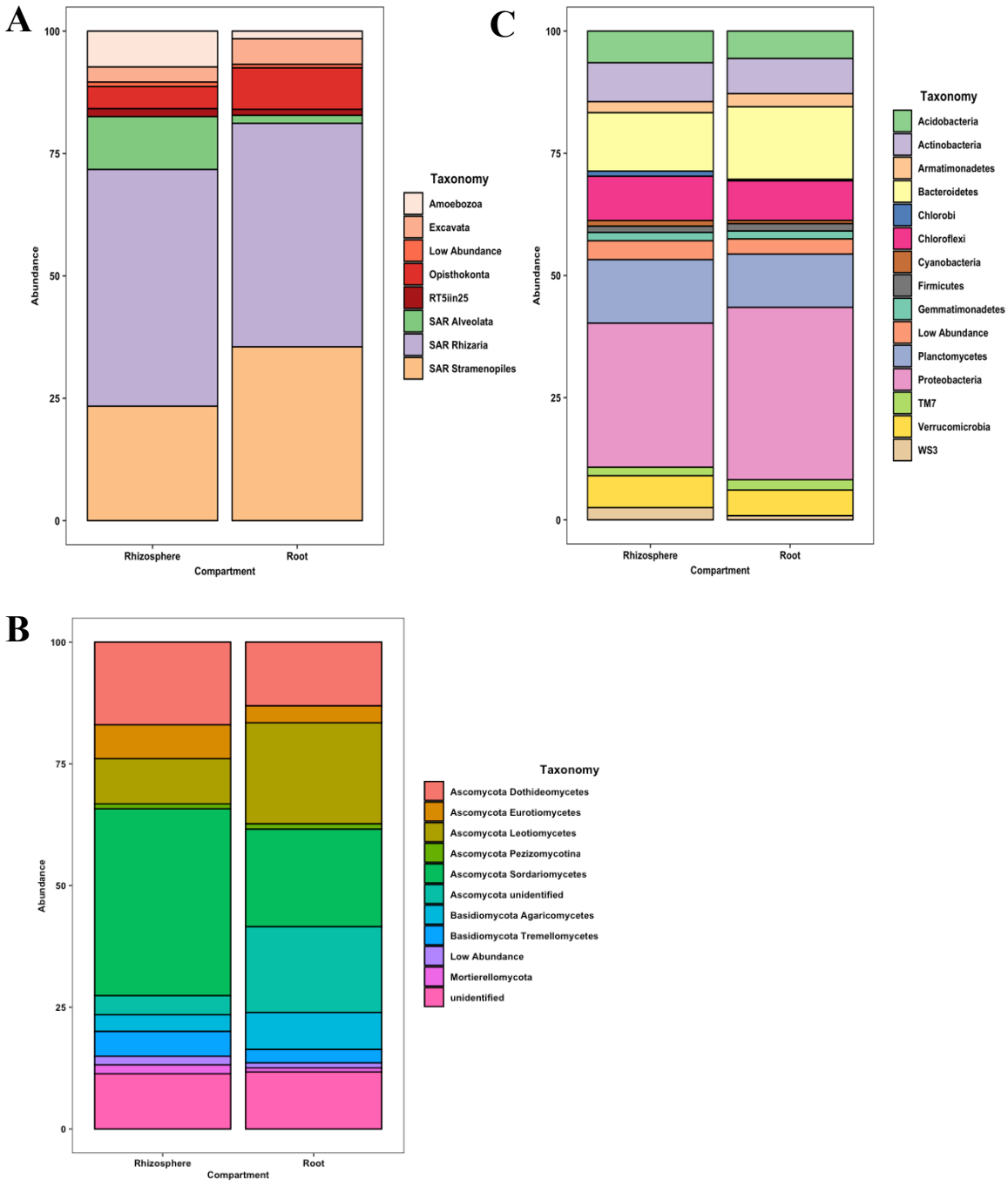

Figure 2.7 Stacked bar plots of bacterial (A), fungal (B) and protist (C) community relative abundances in the root and rhizosphere soil microbiomes. *Data are representative of mean relative abundances of bacterial communities at the phylum level and fungal and protist communities at the Phylum and Class level (Ascomycota & SAR). Statistical analysis was carried out using Kruskal-Wallis test with Dunn's Test post-hoc. N = 84.*

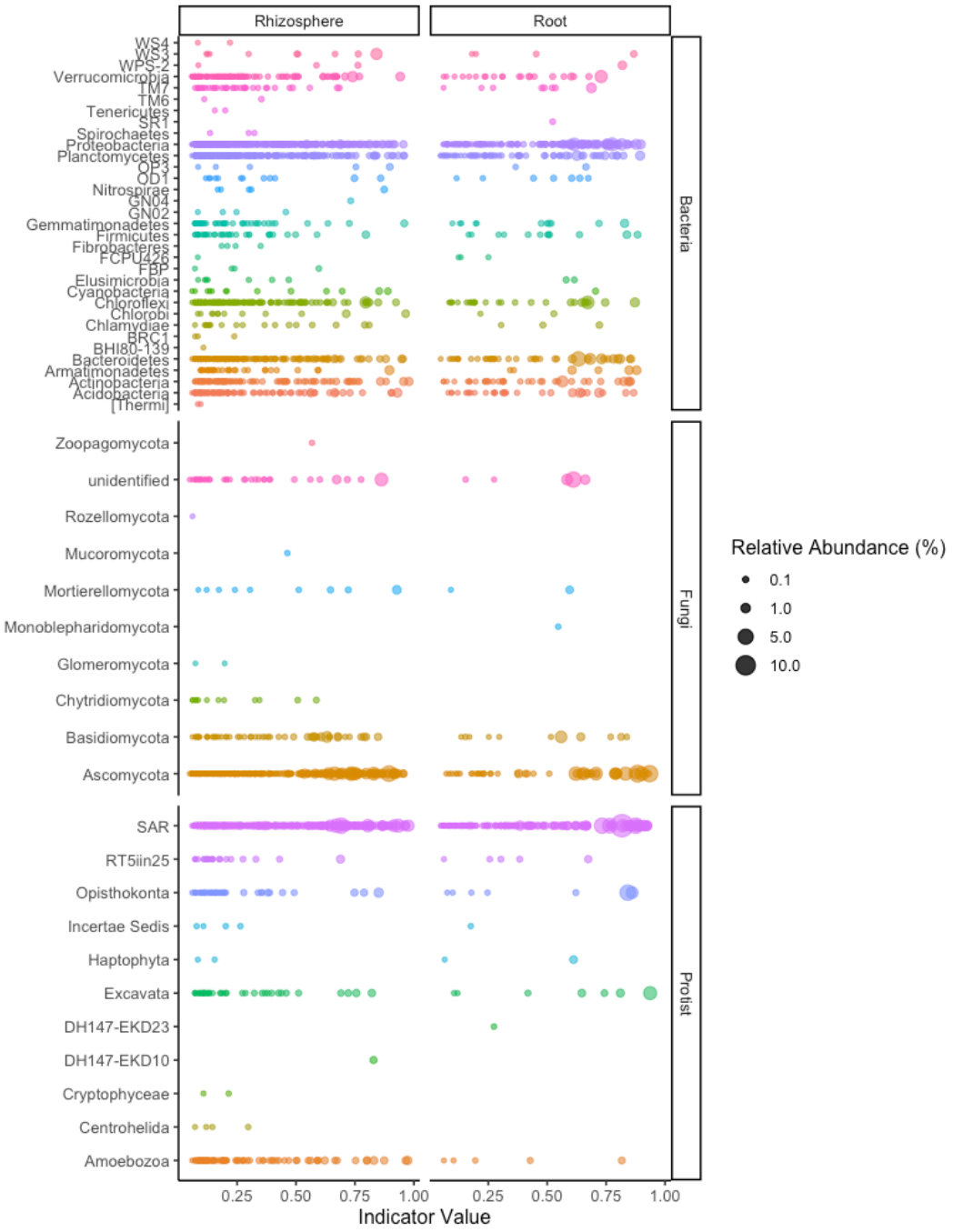

Figure 2.8 Significant indicator species of bacterial, fungal and protist root and rhizosphere soil communities. *Data is representative of significant ($p < 0.05$) OTUs identified by Indicator Analysis (nperm = 999). Significant OTUs are grouped by taxonomic identity at the Phyla level (Ascomycota & SAR split into Class) and ordered by strength of indicator value. Dot size representative of relative abundance of significant indicator OTUs. N = 84.*

Indicator analysis was also conducted and identified many specific bacterial, fungal and protist OTUs that were significantly indicative of either root or rhizosphere soil compartments (**Figure 2.8**). For bacterial communities, 412 OTUs were significantly indicative of the root compartment, with 1564 OTUs indicative of the rhizosphere. For fungal communities, 98 OTUs were indicative of the root compartment, with 520 OTUs indicative of the rhizosphere. Lastly, for protist communities, 153 OTUs were indicative of the root compartment, and 687 OTUs indicative of the rhizosphere. These significantly indicative (or fidelitous) OTUs ranged in their strength of fidelity to either compartment with the strongest indicator OTUs having an indicator value of ≥ 0.8. The top indicator species of the root and rhizosphere communities were from a range of taxa; bacteria OTU74 ((f) Sinobacteraceae), fungi OTU20 ((p) Ascomycota) and protist OTU74 (*Neobodo curvifilus*) were the strongest indicators of the root compartment whereas, bacteria OTU39 ((f) Gaiellales), fungi OTU116 ((f) Hypocreacea) and protist OTU35 ((s) Uncultured Eimeriidae) were the top indicators of rhizosphere soil. (The top five indicator species for root and rhizosphere community can be found in **Table 2.5**).

Within the root and rhizosphere compartments, no plant genotype effect was observed upon the community composition at the Phylum (and Class) level for bacterial, fungal or protists communities (**Figure 2.9**). However, Indicator analysis did identify several OTUs belonging to a range of bacterial, fungal and protist taxa which were significant indicators of each genotype (**Figure 2.10 & 2.11**). These indicator OTUs were found to have less strong indicator values compared with those identified in the compartmental analysis with the strongest genotype indicator OTUs between 0.2 and 0.6 compared to the strongest compartment indicator OTUs ≥ 0.8 (**Table 2.6, 2.7 & 2.8**). (The top five indicator species for genotype root and rhizosphere soil communities can be found in **Table 2.6, 2.7 & 2.8**).

Table 2.5 Top 5 significant indicator species of bacterial, fungal and protist root and rhizosphere soil communities.

					relative abundance (%)	
kingdom	**compartment**	**otu**	**taxonomy**	**ind val**	**rhizosphere soil**	**root**
bacteria	root	OTU41	(f) Sinobacteraceae	0.897	0.103	0.897
		OTU18	(c) OM190	0.894	0.084	0.916
		OTU133	(g) Paenibacillus	0.883	0.106	0.894
		OTU23	(c) OPB50	0.879	0.121	0.879
		OTU29	(o) Myxococcales	0.878	0.122	0.878
				ind val	**rhizosphere soil**	**root**
	rhizosphere soil	OTU39	(f) Gaiellaceae	0.979	0.979	0.021
		OTU48	(c) SJA-28	0.965	0.965	0.035
		OTU171	(c) Gemm-3	0.959	0.959	0.041
		OTU85	(f) Gemmataceae	0.959	0.959	0.041
		OTU5536	(f) Micrococcaceae	0.957	0.957	0.043
				ind val	**rhizosphere soil**	**root**
fungi	root	OTU 20	(p) Ascomycota	0.935	0.065	0.935
		OTU 1036	(c) Sordariomycetes	0.930	0.011	0.989
		OTU 123	(s) *Thelebolus globosus*	0.904	0.062	0.938

		OTU 30	(p) Ascomycota	0.903	0.097	0.903
		OTU 10	(o) Helotiales	0.883	0.117	0.883
				ind val	**rhizosphere soil**	**root**
	rhizosphere soil	OTU116	(f) Hypocreacea	0.957	0.957	0.043
		OTU87	(s) *Arthrographis longispora*	0.953	0.965	0.035
		OTU53	(g) Oidiodendron	0.933	0.944	0.056
		OTU47	(s) *Mortierella minutissima*	0.928	0.939	0.061
		OTU90	(s) *Trichoderma evansii*	0.924	0.947	0.053
				ind val	**rhizosphere soil**	**root**
protist	root	OTU74	(s) *Neobodo curvifilus*	0.937	0.063	0.937
		OTU243	(s) *Allantion sp. CCAP 1906/1*	0.923	0.041	0.959
		OTU4445	(c) Pythium	0.922	0.078	0.922
		OTU7156	(c) Pythium	0.921	0.044	0.956
		OTU836	(c) Glissomonadida	0.909	0.091	0.909
				ind val	**rhizosphere soil**	**root**
	rhizosphere soil	OTU35	(g) Cercomonas	0.977	0.977	0.023
		OTU242	(s) *Vermamoeba vermiformis*	0.974	0.986	0.014
		OTU373	(s) *Acanthamoeba sp. CDC: V621*	0.966	0.977	0.023
		OTU94	(g) uncultured eukaryote	0.965	0.977	0.023
		OTU96	(s) *Thaumatomonadida environmental sample*	0.960	0.984	0.016

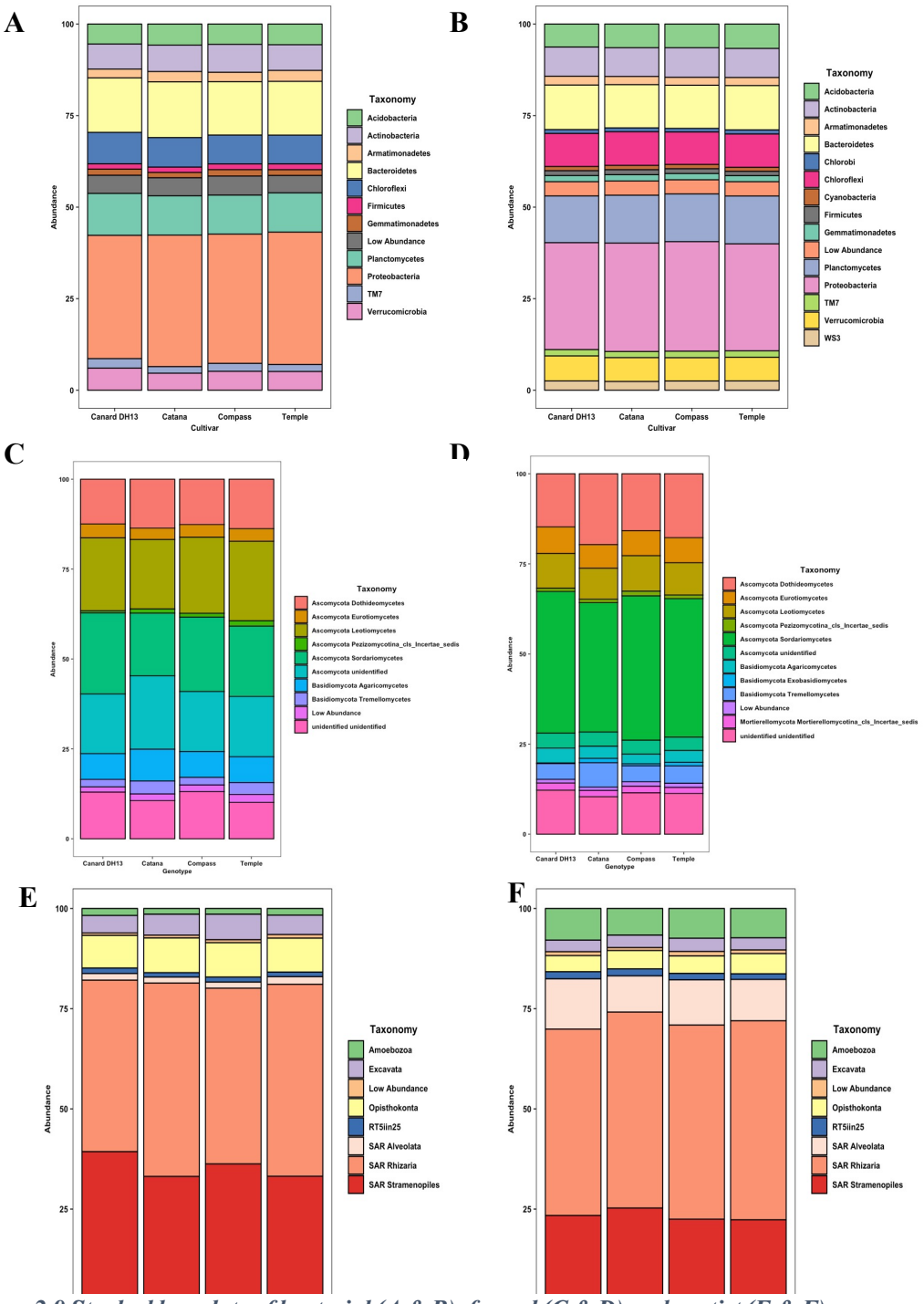

Figure 2.9 Stacked bar plots of bacterial (A & B), fungal (C & D) and protist (E & F) community relative abundances in the root (A, C & E) and rhizosphere soil (B, D & F) microbiomes OSR genotypes. *Data are representative of mean relative abundances of bacterial communities at the phylum level and fungal and protist communities at the Phylum and Class level (Ascomycota & SAR). Statistical analysis was carried out using Kruskal-Wallis test with Dunn's Test post-hoc. N = 84.*

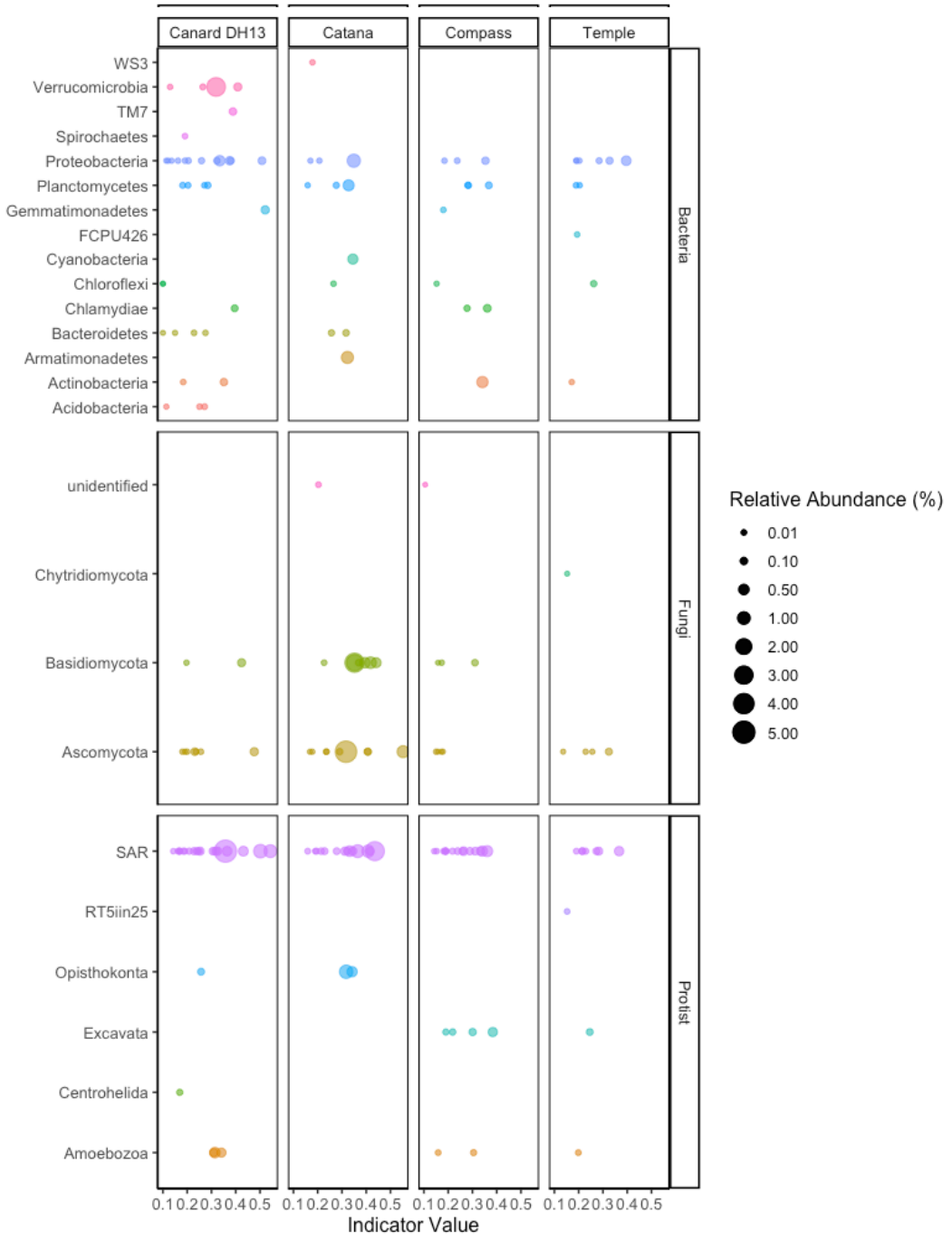

Figure 2.10 Significant indicator species of bacterial, fungal and protist rhizosphere soil communities of OSR genotypes. *Data is representative of significant ($p < 0.05$) OTUs identified by Indicator Analysis (nperm = 999). Significant OTUs are grouped by taxonomic identity at the Phyla level (Ascomycota & SAR split into Class) and ordered by strength of indicator value. Dot size representative of relative abundance of significant indicator OTUs. N = 84.*

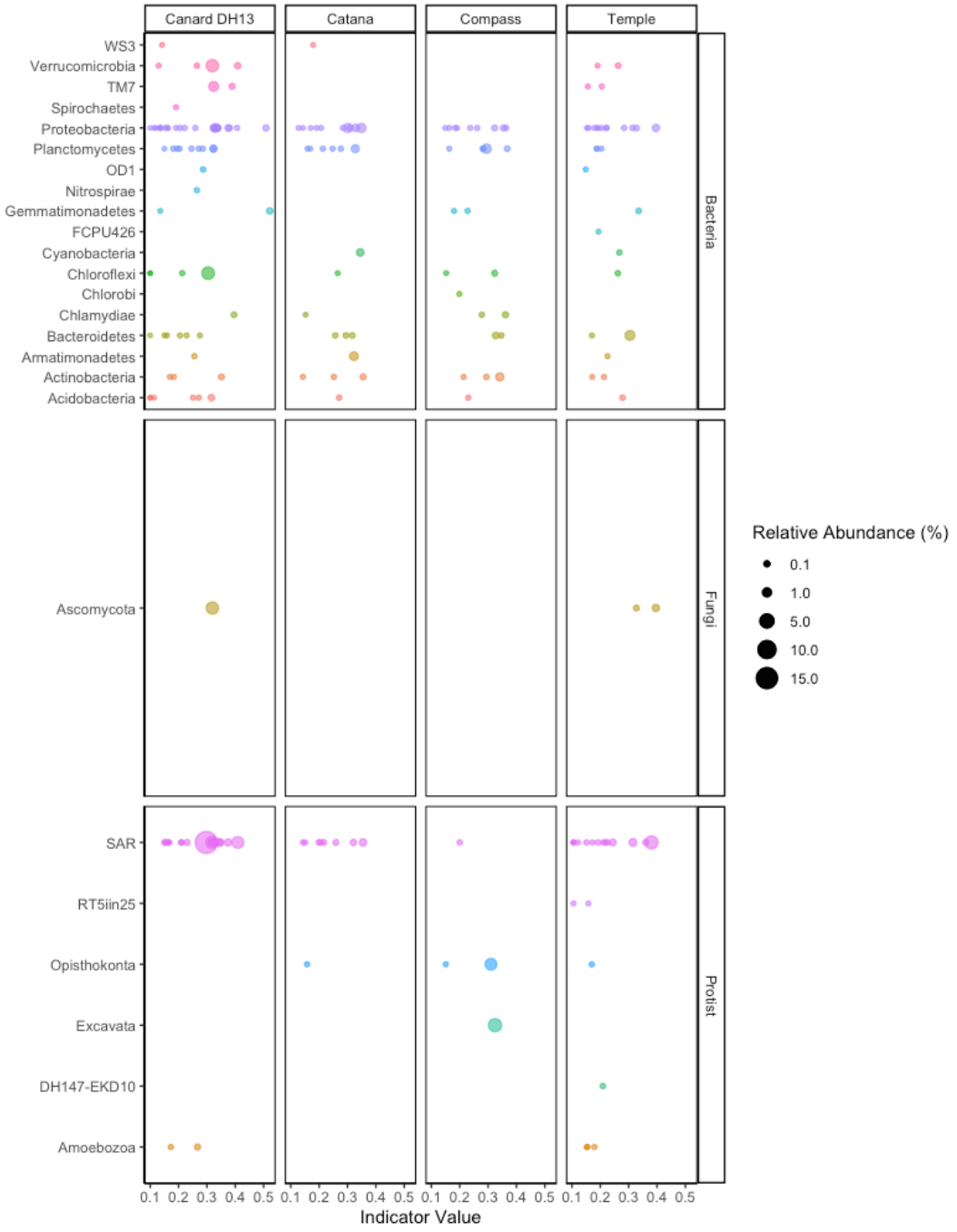

Figure 2.11 Significant indicator species of bacterial, fungal and protist root communities of OSR genotypes. *Data is representative of significant ($p < 0.05$) OTUs identified by Indicator Analysis (nperm = 999). Significant OTUs are grouped by taxonomic identity at the Phyla level (Ascomycota & SAR split into Class) and ordered by strength of indicator value. Dot size representative of relative abundance of significant indicator OTUs. N = 84*

Table 2.6 Top 5 significant indicator species of bacterial root and rhizosphere soil communities in each OSR genotype.

compartment	genotype	otu	taxonomy	indval	relative abundance (%)			
					canard	catana	compass	temple
rhizosphere soil	Canard	OTU292	(f) Ellin5301	0.522	**0.580**	0.068	0.116	0.237
		OTU470	(o) BD73	0.508	**0.635**	0.101	0.124	0.140
		OTU527	(o) Pedosphaerales	0.409	**0.454**	0.132	0.228	0.187
		OTU243	(g) Candidatus Rhabdochlamydia	0.396	**0.495**	0.161	0.184	0.161
		OTU163	(p) TM7	0.388	**0.777**	0.035	0.059	0.129
	Catana	OTU41	(f) Sinobactereaceae	0.349	0.137	**0.349**	0.253	0.261
		OTU74	(o) MLE1-12	0.345	0.180	**0.345**	0.263	0.212
		OTU78	(o) W2101	0.328	0.191	**0.328**	0.307	0.174
		OTU51	(f) Chthonomonadaceae	0.323	0.179	**0.323**	0.226	0.272
		OTU338	(f) Chitinophagaceae	0.317	0.193	**0.416**	0.196	0.196
	Compass	OTU336	(o) DH61	0.368	0.081	0.280	**0.406**	0.232
		OTU6835	(o) Chlamydiales	0.361	0.197	0.143	**0.399**	0.261
		OTU529	(o) Myxococcales	0.354	0.200	0.267	**0.354**	0.180
		OTU77	(f) Nocardioidaceae	0.341	0.240	0.218	**0.341**	0.201
		OTU341	(g) Gemmata	0.283	0.094	0.253	**0.371**	0.282
	Temple	OTU299	(f) Sinobactereaceae	0.396	0.168	0.249	0.187	**0.396**
		OTU57	(g) Labrys	0.327	0.272	0.115	0.231	**0.382**
		OTU932	(g) Rhodobacter	0.284	0.116	0.193	0.193	**0.497**
		OTU2256	(c) TK10	0.262	0.198	0.236	0.173	**0.393**
		OTU261	(o) WD2101	0.204	0.000	0.000	0.143	**0.857**
	Canard	OTU292	(f) Ellin5301	0.522	**0.580**	0.068	0.116	0.237

root		OTU470	(o) BD7-3	0.508	**0.635**	0.101	0.124	0.140
		OTU527	(o) Pedosphaerales	0.409	**0.454**	0.132	0.228	0.187
		OTU1082	(o) Rhizobiales	0.406	**0.677**	0.046	0.230	0.046
		OTU243	(g) Candidatus Rhabdochlamydia	0.396	**0.495**	0.161	0.184	0.161
	Catana	OTU1056	(f) C111	0.355	0.212	**0.414**	0.213	0.161
		OTU41	(f) Sinobacteraceae	0.349	0.137	**0.349**	0.253	0.261
		OTU74	(o) MLE1-12	0.345	0.180	**0.345**	0.263	0.212
		OTU210	(c) ZB2	0.336	0.133	**0.336**	0.278	0.254
		OTU5011	(f) Rhodocyclaceae	0.329	0.175	**0.329**	0.248	0.248
	Compass	OTU336	(o) DH61	0.368	0.081	0.280	**0.406**	0.232
		OTU565	(o) Myxococcales	0.363	0.135	0.128	**0.545**	0.192
		OTU6835	(o) Cytophagales	0.361	0.197	0.143	**0.399**	0.261
		OTU529	(o) Myxococcales	0.354	0.200	0.267	**0.354**	0.180
		OTU175	(f) Chitinophagaceae	0.347	0.155	0.211	**0.486**	0.148
	Temple	OTU299	(f) Sinobacteraceae	0.396	0.168	0.249	0.187	**0.396**
		OTU378	(f) Ellin5301	0.335	0.181	0.182	0.246	**0.391**
		OTU57	(g) Labrys	0.327	0.272	0.115	0.231	**0.382**
		OTU100	(f) Sinobactereaceae	0.313	0.208	0.176	0.110	**0.506**
		OTU7	(g) Flavobacterium	0.305	0.222	0.236	0.238	**0.305**

Table 2.7 Top 5 significant indicator species of fungal root and rhizosphere soil communities in each OSR genotype.

compartment	genotype	otu	taxonomy	indval	relative abundance (%)			
					canard	catana	compass	temple
rhizosphere soil	Canard	OTU133	(g) Tetracladium	0.476	**0.714**	0.123	0.120	0.043
		OTU103	(o) Polyporales	0.424	**0.495**	0.183	0.166	0.156
		OTU308	(p) Ascomycota	0.231	**0.971**	0.015	0.000	0.015
		OTU1242	(g) Bradymyces	0.199	**0.598**	0.070	0.156	0.176
		OTU1248	(s) *Sparassis cystidiosa*	0.197	**0.460**	0.092	0.356	0.092
	Catana	OTU1061	(g) Sporobolomyces	0.441	0.052	**0.441**	0.171	0.336
		OTU203	(s) *Torula masonii*	0.407	0.258	**0.407**	0.098	0.238
		OTU192	(g) Paraphoma	0.406	0.151	**0.406**	0.240	0.203
		OTU1624	(s) *Oliodendron rhodogenum*	0.237	0.106	**0.452**	0.176	0.266
		OTU2116	(o) Pleosporales	0.168	0.000	**0.588**	0.216	0.196
	Compass	OTU140	(s) *Soliococcozyma fuscescens*	0.310	0.155	0.240	**0.421**	0.184
		OTU1085	(s) *Pochonia cordycepisociata*	0.178	0.000	0.153	**0.847**	0.000
		OTU1024	(g) Mycena	0.158	0.000	0.000	**1.000**	0.000
		OTU1110	(f) Sympoventuriaceae	0.158	0.000	0.000	**1.000**	0.000
		OTU2404	(p) Unidentified fungi	0.105	0.000	0.000	**1.000**	0.000
	Temple	OTU120	(s) *Fusarium concentricum*	0.324	0.189	0.261	0.209	**0.341**
		OTU2784	(p) Ascomycota	0.256	0.000	0.110	0.121	**0.769**
		OTU3272	(s) *Buergenerula spartinae*	0.229	0.055	0.164	0.181	**0.601**
		OTU3730	(g) Rhizophydium	0.152	0.000	0.200	0.000	**0.800**
		OTU647	(s) *Myrtapenidiella corymbia*	0.136	0.143	0.143	0.000	**0.714**
root	Canard	OTU17	(s) *Phoma crystallifera*	0.319	**0.319**	0.201	0.241	0.239
	Temple	OTU299	(s) *Coniochaeta lignacola*	0.396	0.168	0.249	0.187	**0.396**

	OTU57	(o) Eurotiales	0.327	0.272	0.115	0.231	**0.382**

Table 2.8 Top 5 significant indicator species of protist root and rhizosphere soil communities in each OSR genotype

compartment	genotype	otu	taxonomy	indval	Relative abundance (%)			
					canard	catana	compass	temple
rhizosphere soil	Canard	OTU415	(s) uncultured plasmodiophorid	0.543	**0.600**	0.157	0.159	0.084
		OTU116	(s) uncultured marine eukaryote	0.502	**0.502**	0.134	0.154	0.210
		OTU340	(s) uncultured cercozoan	0.431	**0.477**	0.166	0.192	0.166
		OTU418	(s) *Exocolpoda augustini*	0.364	**0.382**	0.217	0.120	0.281
		OTU31	(g) uncultured Eimeriidae	0.358	**0.358**	0.228	0.249	0.165
	Catana	OTU47	(g) uncultured stramenopile	0.435	0.128	**0.435**	0.100	0.336
		OTU350	(s) *Hyaloperonospora parasitica*	0.415	0.026	**0.872**	0.051	0.051
		OTU78	(s) *Paracercomonas paralaciniaegerens*	0.407	0.098	**0.407**	0.214	0.281
		OTU45	(g) Cercomonadida environmental sample	0.365	0.150	**0.365**	0.233	0.253
		OTU174	(f) Chrysophyceae	0.350	0.246	**0.350**	0.207	0.197
	Compass	OTU74	(s) *Neobodo curvifilus*	0.384	0.157	0.297	**0.384**	0.162
		OTU235	(s) uncultured Oxytrichidae	0.361	0.263	0.126	**0.361**	0.250
		OTU291	(s) *Hypotrichia sp. I-99*	0.341	0.154	0.268	**0.358**	0.221
		OTU193	(s) *Eocercomonas sp. HFCC 909*	0.333	0.175	0.289	**0.368**	0.167
		OTU461	(g) Halteria	0.312	0.244	0.189	**0.344**	0.222
	Temple	OTU266	(g) Amb-18S-1480	0.367	0.205	0.245	0.165	**0.385**
		OTU141	(g) Paulinella	0.327	0.172	0.239	0.262	**0.327**
		OTU383	(s) *Arcuospathidium muscorum*	0.320	0.249	0.221	0.177	**0.354**
		OTU4445	(c) Pythium	0.283	0.077	0.264	0.264	**0.396**

		OTU728	(g) Cercomonas	0.273	0.151	0.264	0.226	**0.358**
root	Canard	OTU31	(f) Pythium	0.409	**0.409**	0.210	0.203	0.177
		OTU54	(s) *Euglypha rotunda*	0.374	**0.416**	0.151	0.214	0.219
		OTU106	(s) *Cyphoderia amphoralis*	0.348	**0.435**	0.278	0.122	0.165
		OTU1843	(s) *Aphanomyces invadans*	0.344	**0.458**	0.169	0.152	0.220
		OTU161	(g) Cercomonadida environmental sample	0.327	**0.327**	0.212	0.222	0.238
	Catana	OTU254	(g) Cercomonadida environmental sample	0.355	0.183	**0.372**	0.265	0.179
		OTU224	(s) *Paracercomonas oxoniensis*	0.331	0.202	**0.331**	0.214	0.253
		OTU2453	(s) *Pythium capillosum*	0.321	0.213	**0.481**	0.152	0.154
		OTU3464	(s) *Planothidium lanceolatum*	0.259	0.071	**0.454**	0.249	0.226
		OTU780	(s) *Gyromitus sp. HFCC94*	0.217	0.106	**0.505**	0.202	0.186
	Compass	OTU74	(s) *Neobodo curvifilus*	0.325	0.203	0.251	**0.325**	0.221
		OTU92	(s) *Nuclearia moebiusi*	0.310	0.195	0.255	**0.310**	0.240
		OTU2156	(s) *Paracercomonas saepenatans*	0.200	0.160	0.000	**0.840**	0.000
		OTU10663	(s) *Rhynchomonas nasuta*	0.188	0.041	0.039	**0.788**	0.131
		OTU1366	(f) uncultured alveolate	0.151	0.208	0.000	**0.792**	0.000
	Temple	OTU78	(s) *Paracercomonas paralaciniaegerens*	0.381	0.164	0.285	0.171	**0.381**
		OTU259	(g) Ochromonadaceae environmental sample	0.360	0.127	0.121	0.182	**0.570**
		OTU801	(s) *Gregarina chortiocetes*	0.244	0.037	0.035	0.000	**0.928**
		OTU432	(s) *Phytophthora infestans T30-4*	0.226	0.097	0.093	0.093	**0.717**
		OTU1506	(f) uncultured eukaryote	0.218	0.199	0.189	0.151	**0.460**

2.4.3 Relationship of rhizosphere microbiome assembly to OSR biomass and yield.

Analysis of crop biomass and achieved yield was also conducted to assess the relationship between microbiome composition and these key agronomic outcomes. Comparison of the weights of shoot and root systems revealed no significant differences between plant genotypes (**Figure 2.12A**); however, significantly lower yields were observed in the Canard genotype compared to Catana, Compass and Temple (lower yield expected due to Canard genotypic traits) (**Figure 2.12B**). Interestingly, total plant biomass at the microbiome sampling timepoint was not significantly correlated with final achieved yields (**Figure 2.12C**). In addition, variation in the biomass and yield data was considerable for each genotype. Therefore, analysis of biomass and yield data in relation to the microbiome was conducted individually for the root and rhizosphere compartments of each plant genotype. This enabled an observation of whether intra-genotypic variation of biomass and yield data was driven by microbiome composition.

An assessment of whether individual bacterial, fungal or protist taxa (grouped at the Phylum to OTU taxonomic levels) were significantly correlated with biomass and/or yield data, failed to identify any taxa with significant correlations. However, analysis of whether overall microbiome composition (β-diversity) contributed to the variation observed in biomass and yield data (ADONIS Analysis) did identify microbiome composition as a significant driver of plant biomass variation in certain cases (**Table 2.9 & Figure 2.13**). Variation in shoot and root biomass of different genotypes was found to be influenced by bacterial and fungal microbiome β-diversity, with contribution to variation ranging from 5.596 – 6.617 % (**Table 2.9**). Only the bacterial rhizosphere microbiome of Canard was found to contribute to the variation of yield with a contribution of 5.596 % (**Table 2.9**). Ordination of biomass and yield data upon NMDS ordination of microbiome β-diversity enabled visualisation of these contributions to biomass and yield variation with clear gradients observed (**Figure 2.13**).

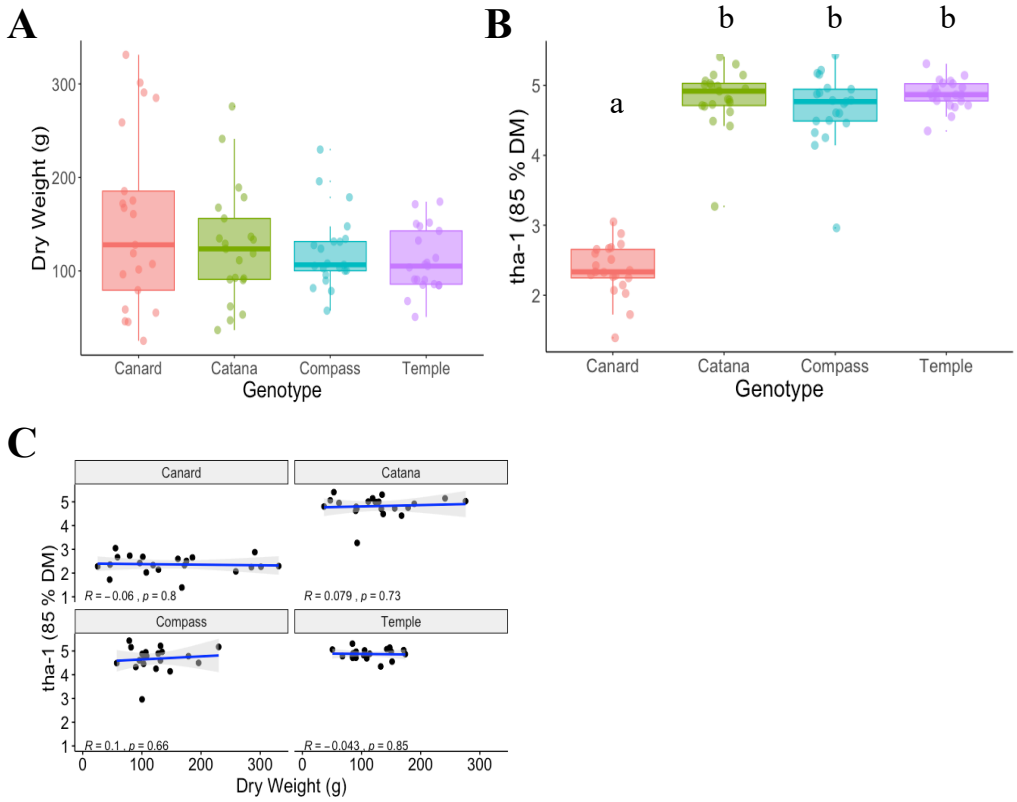

Figure 2.12 OSR yield and biomass data. *Dry weight of sampled plants (A), final achieved yields (85 % DM moisture content) (B) and correlation of biomass and yield. Data are grouped by genotype used in study with n=21 for each cultivar. For A & B data were analysed in R using a Kruskal-Wallis statistical analysis for non-parametric data with a Dunn's Test post-hoc analysis. Groups with significantly different yields ($p < 0.001$) are indicated by different letters. (C) correlation analysis was carried out using the Pearson's correlation test with correlation value (R) and p values denoted on graph N = 21.*

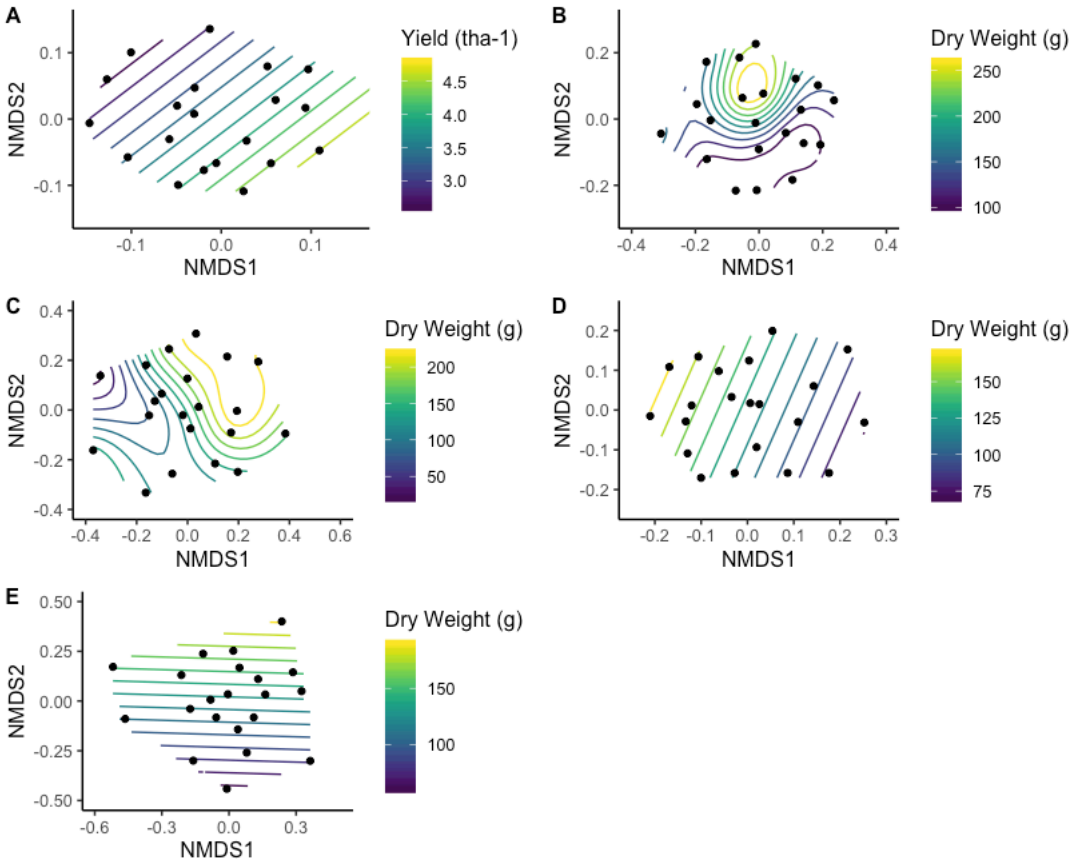

Figure 2.13 Ordination of yield and biomass data as significant explanatory variables of community β-dissimilarity. *Biomass and yield data identified as significant ($p < 0.05$) explanatory variables of β-dissimilarity as identified by ADONIS analysis was ordinated over community NMDS $\beta - dissimilarity$ data. (A) Canard bacterial rhizosphere community (B) Canard fungal rhizosphere community (C) Canard fungal root community D Catana fungal rhizosphere community E Catana fungal root community. N = 21.*

Table 2.9 Significant contributing factors to community β-Diversity as identified by ADONIS analysis.

community	compartment	genotype	main factor	contribution	p-value
bacteria	rhizosphere soil	Canard	Yield	5.596 %	0.015
fungi	rhizosphere soil	Canard	Plant weight	6.618 %	0.003
		Catana	Plant weight	5.917 %	0.018
	root	Canard	Plant weight	5.844 %	0.037
		Catana	Plant weight	6.187 %	0.012

2.4.4 Microbial inter-taxon co-occurrence networks

Inter-taxon co-occurrence networks were created to assess occurrence relationships between the bacterial, fungal and protist taxa described in this study. Taxa found to have significant co-occurring/excluding interactions were identified with relationships satisfying the correlation value threshold (cor < -0.4 & cor > 0.4) being retained and constructed into a network. From this, analysis of network composition, structure and statistics was undertaken. Overall root and rhizosphere soil networks were constructed by combining genotype samples to observe inter-taxon relationships which are common amongst individual OSR genotypes. Subsequently, inter-taxon co-occurrence networks were constructed for individual genotype root and rhizosphere soil microbiomes to determine genotype specific signatures.

2.4.4.1 Combined root and rhizosphere soil inter-taxon co-occurrence networks

Compositionally and structurally different networks were identified for the combined root and rhizosphere soil microbiomes of OSR (**Figure 2.14 & Table 2.10**). Regarding composition, a similar number of nodes were found in the root and rhizosphere soil networks (98 and 101 respectively) (**Table 2.10**). However, nodes

differed in their taxonomic composition between the networks. The root network was predominantly composed of bacterial taxa (42.57 %) followed by fungal (38.61 %) and protist (18.81 %) taxa. In comparison, the rhizosphere soil network was predominantly composed of fungal taxa (61.22 %) followed by bacterial (29.59 %) and protist (9.18 %) taxa (**Table 2.10**). Comparison of node taxa composition also identified differences in the representation of fungal, bacterial and protist taxa at the Class level (**Figure 2.14)**

A

B

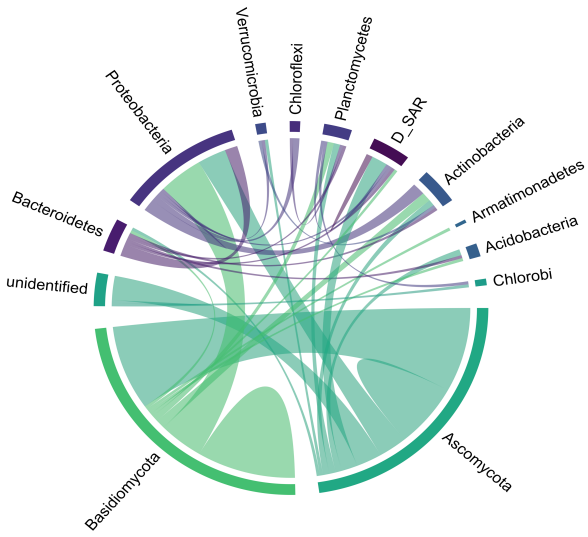

Figure 2.14 Inter-taxon co-occurrence networks of root (A) and rhizosphere soil (B) microbiomes. *Individual Taxa are grouped by Phylum with significant co-occurrence/exclusion relationships between taxa represented by chord. Chords are coloured to easily differentiate each chord and are not indicative of any metadata. Networks were created using FastSpar with analysis & visualisation in R using the Igraph & Circlize packages (respectively). Threshold=0.4, n=84.*

Table 2.10 Network statistics of combined root and rhizosphere soil inter-taxon co-occurrence networks.

Network statistic	rhizosphere soil	root
node number	98	101
edge number	158	121
number of +ve edges (%)	143 (88.61 %)	98 (89.99 %)
number of -ve edges (%)	18 (11.39 %)	23 (19.01 %)
mean degree	3.224	2.396
density	0.033	0.024
transitivity	0.362	0.271
number of clusters	15	21
mean cluster size	6.533	4.81
max. cluster size	20	20
min. cluster size	2	2
modularity	0.564	0.739
mean node betweenness centrality	88.541	111.366
protist number (% of network)	9 (9.18 %)	19 (18.81 %)
fungi number (% of network)	60 (61.22 %)	39 (38.61 %)
bacteria number (% of network)	29 (29.59 %)	43 (42.57 %)
intra-kingdom edges (% of edges)	109 (68.99 %)	97 (80.17 %)
inter-kingdom edges (% of edges)	49 (31.01 %)	24 (19.83 %)
number of b:b edges (% of edges)	18 (11.39 %)	47 (38.84 %)

number of b:f edges (% of edges)	40 (25.32 %)	2 (1.65 %)
number of b:p edges (% of edges)	3 (1.90 %)	16 (13.22 %)
number of f:f edges (% of edges)	89 (56.33 %)	39 (32.23 %)
number of f:p edges (% of edges)	6 (3.80 %)	6 (4.96 %)
number of p:p edges (% of edges)	2 (1.30 %)	11 (9.09 %)

A variety of structural differences in the root and rhizosphere networks were also identified in relation to network connectivity, connection type and overall structure. The rhizosphere soil network was more connected (Edge No.), denser (Mean Node Degree/Density) and less modular (Modularity) compared to the root network (**Table 2.10**). Regarding connection type, difference in the proportions of positive (co-occurring) and negative (co-excluding) edges was also found with less positive edges in the rhizosphere (88.61 %) compared to the root network (89.99 %), however, the predominance of positive edges was identified in both networks. In addition, an examination of which taxa were connected by network edges identified a predominance of intra-taxon connections in both root and rhizosphere networks (**Table 2.10**). However, in the root network bacteria to bacteria connections were the most represented edge type (38.84 %) whereas in the rhizosphere network fungi to fungi connections were the most represented (56.33 %) (**Table 2.10**).

Analysis of networks was also carried out at the node level (OTU level) with specific roles being assigned for each node. These roles are based on two network metrics namely, 'Within Module Degree' (an index value relating to the level of connection a node has within its module) and 'Among Module Connectivity' (an index value relating to the level of connection a node has between different modules) with thresholds then being applied to assign one of seven potential network roles. These roles include three 'hub' node roles (Network Hubs, Module Hubs and Provincial Hubs) and four 'other' node roles (Kinless nodes, Connector nodes, Peripheral and Ultra-peripheral nodes). In the root and rhizosphere soil networks the majority of each network's nodes represented OTUs unique to that network (rhizosphere soil = 71.43 % and root = 72.28 %). However, when examining the roles of the most connected nodes (Within Module Degree > 1 and Among Module Connectivity > 0.25), in the rhizosphere network the eight most connected nodes were also present in the root

network, whereas of the five most connected nodes in the root network three were unique and two were shared with the rhizosphere network (**Table 2.12**). Despite these nodes being shared between the networks, their roles differ in each network with lower levels of connectivity in the opposing network (**Table 2.12**). Regarding taxonomy, the top five most highly connected rhizosphere soil nodes were fungi and one bacterium, with the most connected node (a Module Hub) identified as the fungus *Vishniacozyma victoriae*. In the root network, three of the five highly connected nodes were fungi and two bacteria. The most connected node (a Peripheral Hub) was a bacterium identified as a Sinobacteriaceae. These network role assignments also reflected the more modular nature of the root network compared to the denser rhizosphere soil network. Three of the top five highly connected OTUs in each network were present in both networks, however, these OTUs shifted roles between networks (**Figure 2.15**). This was observed in the majority of OTUs shared between root and rhizosphere soil networks (**Figure 2.15**).

Table 2.11 Highly connected taxa (as identified by network role analysis) in combined root and rhizosphere inter-taxon co-occurrence networks.

Network	node id	taxonomy	network role	
root			**root**	**rhizosphere soil**
	B_OTU164	(f) Sinobacteriaceae	Peripheral Hub	Absent
	F_OTU26	(s) *Trichoderma spirale*	Peripheral Node	Ultra-peripheral Node
	F_OTU29	(g) Glarea	Peripheral Node	Ultra-peripheral Node
	B_OTU991	(o) BD7-3	Peripheral Node	Ultra-peripheral Node
	F_OTU1061	(o) Entylomatales	Peripheral Node	Absent
			rhizosphere soil	**root**
rhizosphere soil	F_OTU31	(s) *Vishniacozyma victoriae*	Module Hub	Ultra-peripheral Node
	F_OTU180	(s) *Scopulariopsis cordiae*	Connector Node	Absent
	F_OTU91	(o) Entylomatales	Peripheral Node	Peripheral Node
	B_OTU9	(o) Rhizobiales	Peripheral Node	Ultra-peripheral Node
	F_OTU475	(o) Tremellales	Peripheral Node	Absent

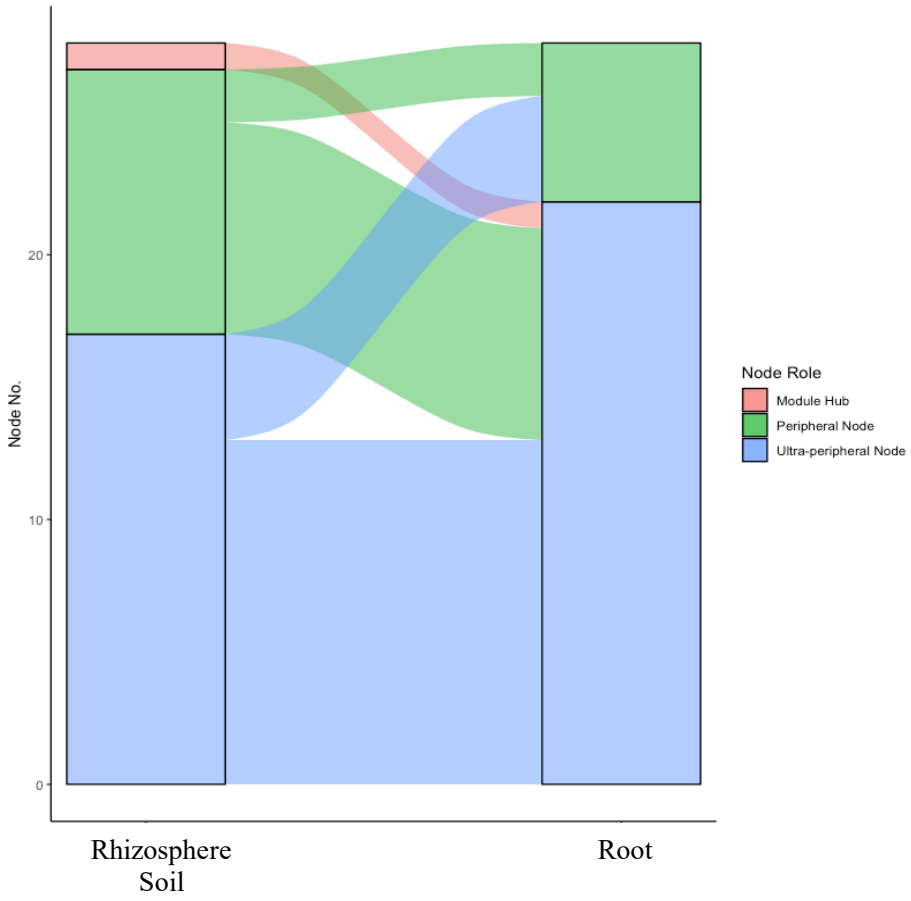

Figure 2.15 Shifting node network roles between nodes shared between root and rhizosphere soil networks. *Data is representative of nodes shared between rhizosphere soil and root networks, with alluvia indicative of shifting node roles in opposing network. Network roles are indicated by bar and alluvia colour.*

2.4.4.2 Genotype specific inter-taxon co-occurrence networks

Compositionally and structurally different networks were identified between plant genotypes in both the root and rhizosphere soil compartments (**Figure 2.16 & 2.17**). Regarding composition, a higher node number was found in the root and rhizosphere soil networks of Canard and Temple compared to Catana and Temple (**Table 2.13** & **Table 2.14**). For root networks, bacteria comprised the largest proportion of nodes, then fungi and protist for all genotypes (**Table 2.13**). This was reflective of the overall root network constructed from all genotype samples (**Table 2.11**). However, for rhizosphere soil networks this was only true for Canard, Catana

and Temple with fungi predominating in the Compass (**Table 2.14**). This is at odds with the combined rhizosphere soil network where fungi predominated (**Table 2.11**). Comparison of node taxa composition also identified differences in the representation of fungal, bacterial and protist taxa at the Class level in both root and rhizosphere genotype microbiomes (**Figure 2.16 & 2.17**).

A variety of structural differences in the root and rhizosphere soil networks were also identified in relation to network connectivity, connection type and overall structure. Co-occurring or 'positive' connections comprised most connections in all genotype rhizosphere soil root networks, however, the proportion was lower than that identified in combined rhizosphere soil and root networks (**Table 2.11, Table 2.13 & Table 2.14**). Structural differences in terms of connectivity, density and modularity also varied between genotype networks in both the root and rhizosphere soils (**Table 2.13 & Table 2.14**).

OTU level analysis of root and rhizosphere soil genotype networks also identified distinct networks for each genotype. Despite most nodes being shared between genotype networks (**Figure 2.18**), the roles of these nodes differed between each genotype network (**Figure 2.19A & B**). This is true of all network roles from the most highly connected Network Hub nodes (**Table 2.15 & Table 2.16**) to the least connected Ultra-Peripheral nodes. Taxonomic differences in the most connected 'Network Hub' nodes included unique (Network Hub in only one genotype) taxa from the bacterial, fungal and protist taxonomic classifications (**Table 2.15 & Table 2.16**).

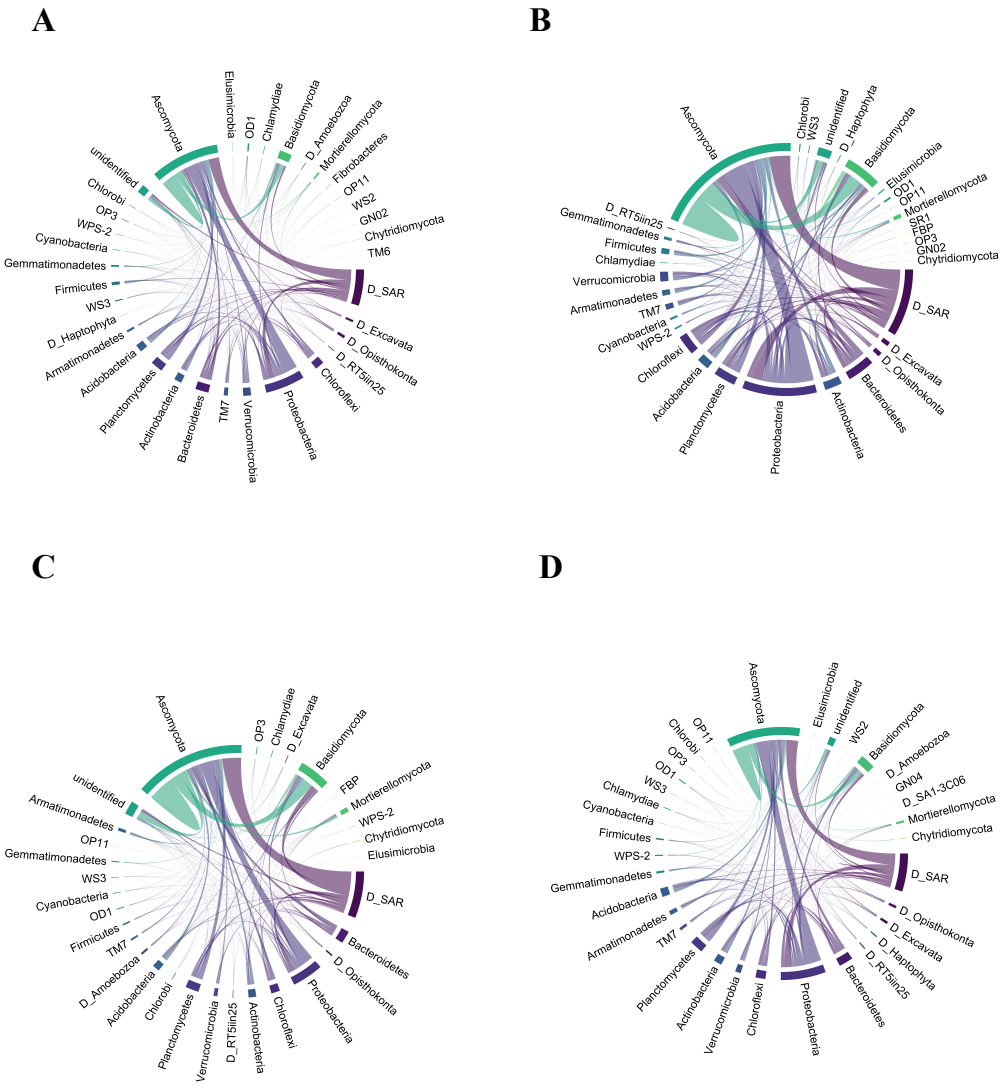

Figure 2.16 Inter-taxon co-occurrence networks of root microbiomes between genotypes. A) *Canard,* ***B)*** *Catana,* ***C)*** *Compass,* ***D)*** *Temple. Individual Taxa are grouped by Phylum with significant co-occurrence/exclusion relationships between taxa represented by chords. Networks were created using FastSpar with analysis & visualisation in R using the Igraph & Circlize packages (respectively). Threshold=0.4, n=21.*

Table 2.12 Network statistics of each genotype's root inter-taxon co-occurrence network.

Network statistic	canard	catana	compass	temple
node number	559	481	484	528
edge number	2582	1938	1723	2631
number of +ve edges (% of edges)	1356 (52.52 %)	1056 (54.49 %)	881 (51.13 %)	1330 (50.55 %)
number of -ve edges (% of edges)	1226 (47.48 %)	882 (45.51 %)	842 (48.87 %)	1301 (49.45 %)
mean degree	9.238	8.058	7.120	9.966
density	0.017	0.017	0.015	0.019
transitivity	0.192	0.19	0.162	0.217
number of clusters	10	10	10	8
mean cluster size	55.9	48.1	48.4	66
max. cluster size	150	112	128	143
min. cluster size	2	2	2	3
modularity	0.390	0.402	0.007	0.370
mean node betweenness centrality	633.644	564.867	579.390	570.055
protist number (% of network)	94 (16.82 %)	77 (16.01 %)	81 (16.40 %)	85 (16.10 %)
fungi number (% of network)	187 (33.45 %)	170 (35.34 %)	185 (37.45 %)	180 (34.10 %)
bacteria number (% of network)	278 (49.73 %)	234 (48.65 %)	218 (44.13 %)	263 (49.81 %)
intra-kingdom edges (% of edges)	1187 (45.97 %)	871 (44.94%)	846 (49.10 %)	1118 (42.49 %)
inter-kingdom edges (% of edges)	1395 (54.03 %)	1067 (55.06 %)	877 (50.90 %)	1513 (57.51 %)
number of b:b edges (% of edges)	604 (23.39 %)	368 (18.99 %)	342 (19.85 %)	566 (21.52 %)
number of b:f edges (% of edges)	705 (27.30 %)	595 (30.70 %)	483 (28.03 %)	780 (29.65 %)
number of b:p edges (% of edges)	351 (13.59 %)	239 (12.33 %)	135 (7.84 %)	370 (14.06 %)
number of f:f edges (% of edges)	502 (19.44 %)	435 (22.45 %)	425 (24.67 %)	481 (18.28 %)
number of f:p edges (% of edges)	339 (13.13 %)	233 (12.02 %)	259 (15.03 %)	363 (13.80 %)
number of p:p edges (% of edges)	81 (3.14 %)	68 (3.51 %)	79 (4.59 %)	71 (2.70 %)

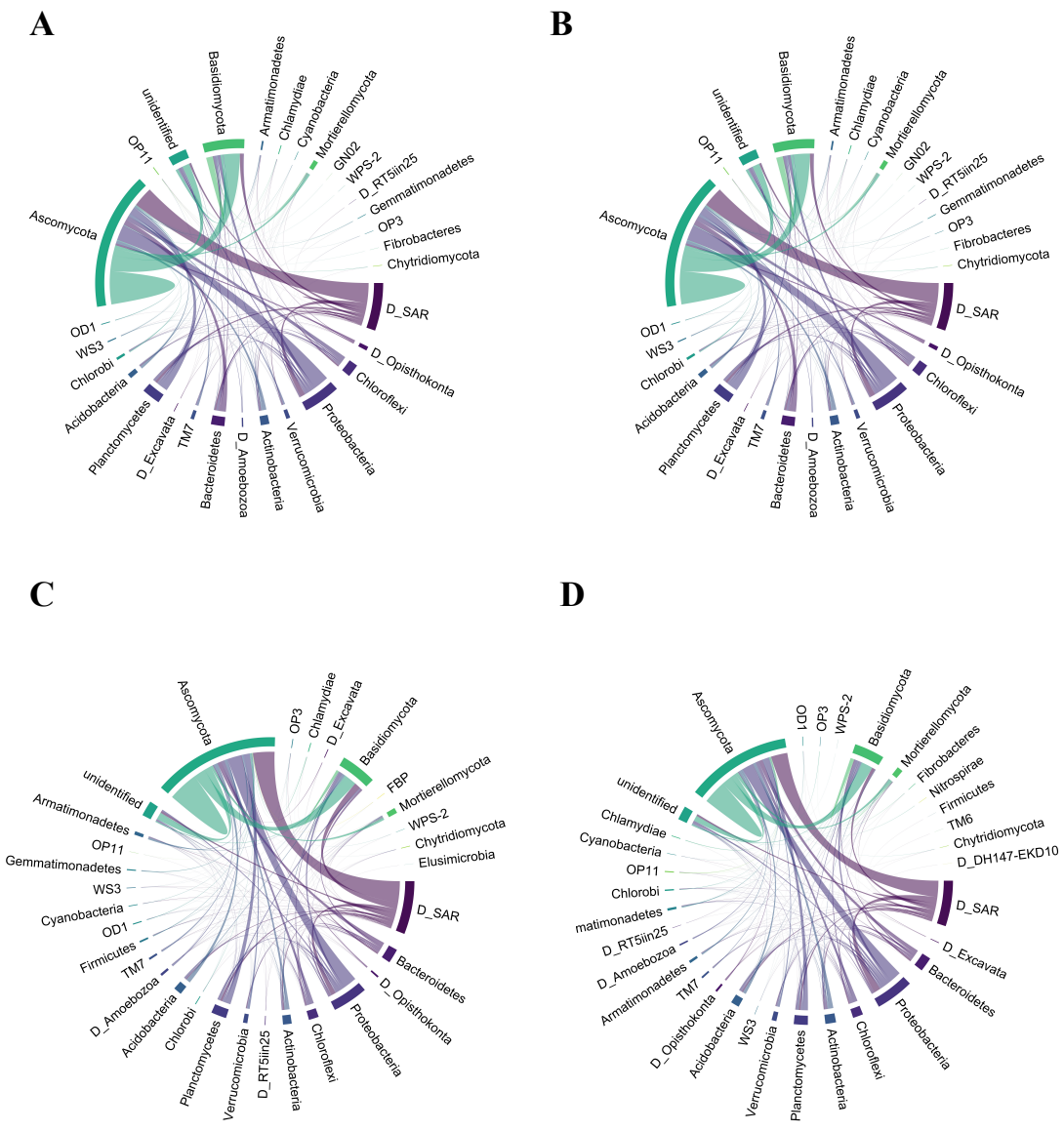

Figure 2.17 Inter-taxon co-occurrence networks of rhizosphere soil microbiomes between genotypes. ***A)*** *Canard,* ***B)*** *Catana,* ***C)*** *Compass,* ***D)*** *Temple. Individual Taxa are grouped by Phylum with significant co-occurrence/exclusion relationships between taxa represented by chords. Networks were created using FastSpar with analysis & visualisation in R using the Igraph & Circlize packages (respectively). Threshold=0.4, n=21.*

Table 2.13 Network statistics of each genotype's rhizosphere inter-taxon co-occurrence network.

Network statistic	canard	catana	compass	temple
node number	584	499	671	589
edge number	1862	1844	2445	2266
number of +ve edges (%)	1007 (54.08 %)	1036 (56.18 %)	1225 (50.10 %)	1224 (54.02 %)
number of -ve edges (%)	855 (45.92 %)	808 (43.82 %)	1186 (48.51 %)	1042 (45.98 %)
mean degree	6.377	7.391	7.288	7.694
density	0.011	0.015	0.011	0.013
transitivity	0.197	0.215	0.189	0.208
number of clusters	14	13	14	16
mean cluster	41.71	38.35	47.92	36.81
max. cluster size	132	110	165	167
min. cluster size	2	2	2	2
modularity	0.427	0.416	0.404	0.411
mean node betweenness centrality	775.392	605.174	823.24	712.306
protist number (% of network)	102 (17.47 %)	94 (18.84 %)	136 (20.27 %)	104 (17.66 %)
fungi number (% of network)	229 (39.21 %)	200 (40.08 %)	286 (42.62 %)	231 (39.22 %)
bacteria number (% of network)	253 (43.32 %)	205 (41.08 %)	249 (37.12 %)	254 (43.12 %)
intra-kingdom edges (% of edges)	889 (47.74 %)	869 (47.13 %)	1024 (41.88 %)	1012 (44.66 %)
inter-kingdom edges (% of edges)	973 (52.26 %)	975 (52.87 %)	1421 (58.12 %)	1254 (55.34 %)
number of b:b edges (% of edges)	262 (14.07 %)	159 (31.86 %)	233 (9.53 %)	288 (12.71 %)
number of b:f edges (% of edges)	696 (37.38 %)	557 (30.21 %)	747 (30.55 %)	685 (30.23 %)
number of b:p edges (% of edges)	90 (4.83 %)	137 (7.43 %)	204 (8.34 %)	222 (9.80 %)
number of f:f edges (% of edges)	595 (31.95 %)	665 (36.06 %)	728 (29.78 %)	665 (29.35 %)
number of f:p edges (% of edges)	187 (10.04 %)	281 (15.24 %)	436 (17.83 %)	347 (15.31 %)
number of p:p edges (% of edges)	32 (1.72 %)	45 (2.44 %)	63 (2.58 %)	59 (2.60 %)

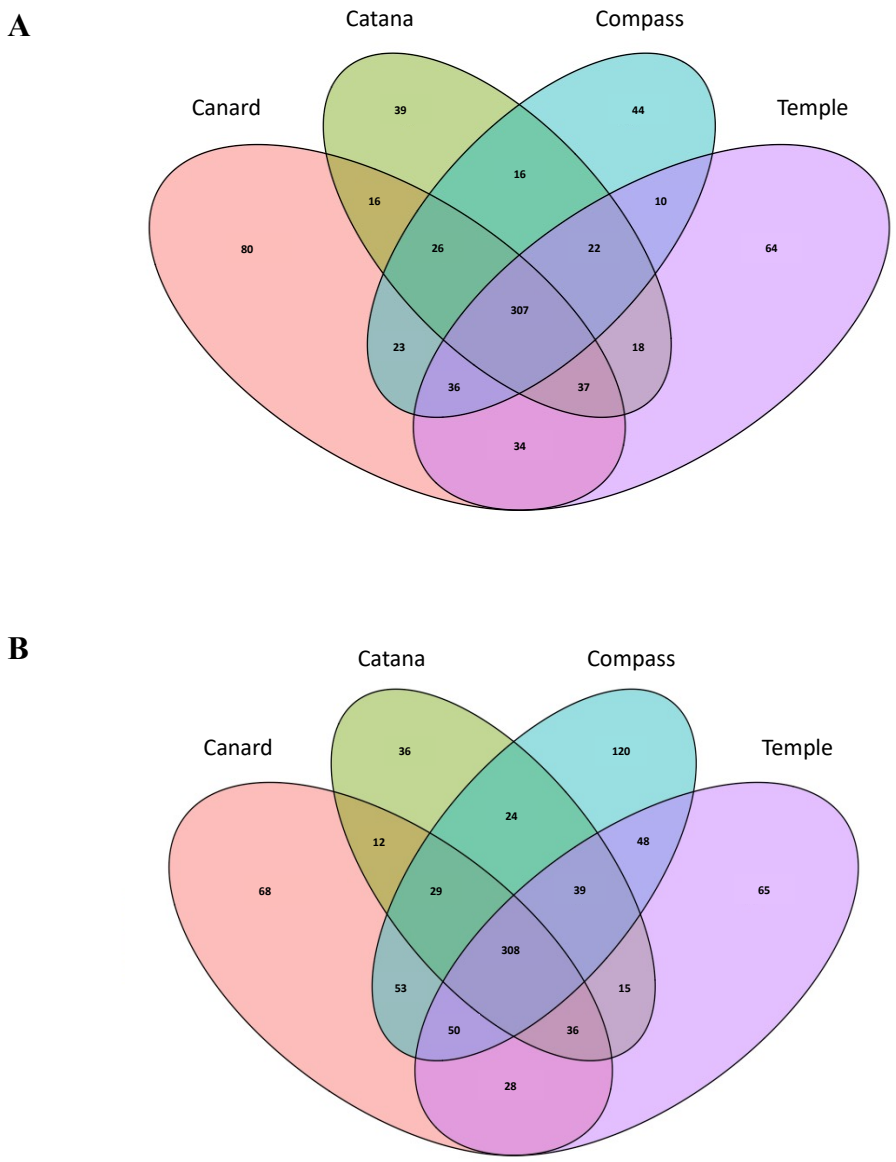

Figure 2.18 Venn diagram of inter-taxon co-occurrence network OTUs shared between genotype root (A) rhizosphere soil (B) networks.

Table 2.14 'Network Hub' taxa (as identified by network role analysis) of each genotype's root inter-taxon co-occurrence networks and network role in opposing genotype networks.

Network genotype	node id	taxonomy	role in opposing network		
			Catana	**Compass**	**Temple**
canard	B_OTU54	(o) FAC87	Connector Node	Connector Node	Peripheral Node
	F_OTU44	(o) Sordariales	Connector Node	Connector Node	Peripheral Hub
			Canard	**Compass**	**Temple**
catana	B_OTU30	(c) TM7-1	Peripheral Hub	Peripheral Node	Peripheral Node
	F_OTU13	(s) *Exophiala equina*	Peripheral Node	Peripheral Node	Connector Node
			Canard	**Catana**	**Temple**
compass	P_OTU4489	(g) Uncultured Eustigmatophyceae	Connector Node	Connector Node	Connector Node
	F_OTU9	(o) Helotiales	Connector Node	Peripheral Node	Peripheral Node
	F_OTU4	(c) Sordariomycetes	Connector Node	Peripheral Node	Connector Node
			Canard	**Catana**	**Compass**
temple	B_OTU950	(f)Rhodospirillaceae	Connector Node	Connector Node	Module Hub
	B_OTU29	(o) Myxococcales	Module Hub	Peripheral Node	Connector Node

Table 2.15 'Network Hub' taxa (as identified by network role analysis) of each genotype's rhizosphere inter-taxon co-occurrence networks and network role in opposing genotype networks.

network genotype	node id	taxonomy	role in opposing network		
			Catana	**Compass**	**Temple**
canard	B_OTU21	(f) Isosphaeraceae	Connector Node	Peripheral Node	Module Hub
	B_OTU5	(c) Gitt-GS-136	Module Hub	Peripheral Node	Peripheral Node
	F_OTU16	(p) Unidentified fungi	Connector Node	Peripheral Node	Connector Node
	F_OTU4	(c) Sordariomycetes	Module Hub	Peripheral Node	Peripheral Node
	F_OTU64	(s) *Itersonilia pannonica*	*Module Hub*	Peripheral Node	Peripheral Node
	F_OTU83	(s) *Trichoderma crassum*	Peripheral Node	Peripheral Node	Peripheral Node
			Canard	**Compass**	**Temple**
catana	P_OTU8	(g) D_uncultured stramenopile	Connector Node	Peripheral Node	Connector Node
	P_OTU54	(g) D_uncultured Eimeriidae	Peripheral Node	Connector Node	Peripheral Node
	F_OTU308	(p) Unidentified Ascomycota	Module Hub	Peripheral Node	Peripheral Hub
			Canard	**Catana**	**Temple**
compass	B_OTU54	(o) FAC87	Peripheral Node	Connector Node	Peripheral Node
	F_OTU12	(f) Lasiosphaeriaceae	Connector Node	Peripheral Node	Peripheral Node
	F_OTU6	(p) Unidentified fungi	Connector Node	Peripheral Node	Network Hub
	F_OTU2138	(p) Unidentified fungi	Module Hub	Connector Node	Peripheral Node
			Canard	**Catana**	**Compass**
temple	F_OTU6	(p) Unidentified fungi	Connector Node	Peripheral Node	Network Hub
	F_OTU46	(s) *Cladobotryum rubrobrunnescens*	Connector Node	Peripheral Node	Peripheral Node

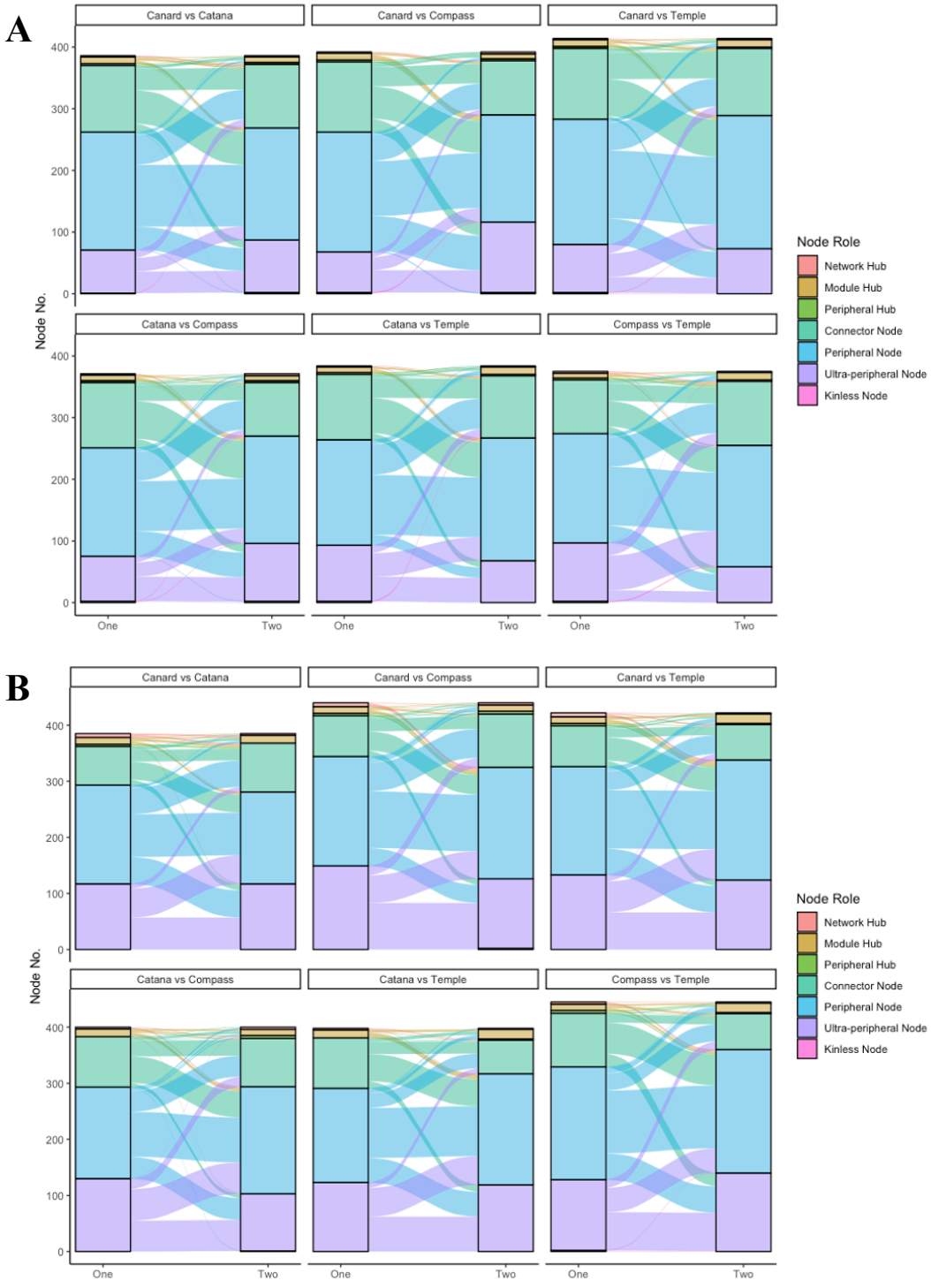

Figure 2.19 ***Shifting node network roles between nodes shared between root (A) and rhizosphere soil (B) networks in OSR genotypes.*** *Data is representative of nodes shared between rhizosphere soil and root networks, with alluvia indicative of shifting node roles in opposing network. Network roles are indicated by bar and alluvia colour.*

2.5 Discussion

The findings of this chapter identified rhizosphere microbiome differentiation at the OTU but not community level between four genotypes of OSR. Community β-diversity metrics showed overlapping communities between genotypes, with a low level of dissimilarity between rhizosphere protist communities of Canard/Catana and Canard/Temple. This dissimilarity was not reflected in terms of community α-diversity or relative abundancies, but at the individual OTU level. Significant indicator OTUs and distinct inter-taxon co-occurrence networks were identified for each genotype, community, and compartment. In addition, a complex interaction between microbiome composition and crop biomass was observed. No individual taxa were linked to crop biomass parameters but, differences in overall community composition of certain genotype communities were significantly associated with crop biomass and yield. Together, these findings indicate that crop genotypes may influence the formation of root and rhizosphere soil microbiomes in the field at the OTU level from similar microbial communities, with different inter-taxon interaction occurring between genotypes and certain OTUs showing fidelity to a particular genotype. In addition, this study shows a relationship between microbial community variation and crop biomass and yield outcomes in certain genotype microbiomes in a real word setting.

2.5.1 OSR genotype and microbiome structure at the community level

The community level analysis presented in this study shows overlapping bacterial, fungal and protist communities in the root and rhizosphere soil of the four OSR genotypes. However, significant dissimilarity based on community beta diversity was identified between Canard / Catana and Canard / Temple rhizosphere protist communities. This dissimilarity was of a low level and not reflected in alpha diversity or relative abundancies (no significant differences were identified in either parameter). This contrasts with other studies of OSR genotypes which identified significant shifts in fungal and bacterial community diversity and relative abundances at the community and individual taxa levels (Bazghaleh et al., 2020, Taye et al., 2020). Taye et al. 2020 identified 67 differentially abundant bacterial taxa which were genotype specific and

proposed that genotypic diversity in OSR may be directed towards the recruitment of specific bacterial taxa. Bazghaleh et al. 2020 identified significant shifts in fungal diversity along genotypic lines with sample year and site also contributing to fungal diversity. Therefore, there is a precedent for genotypes to influence bacterial and fungal root and rhizosphere soil microbiome composition and diversity in OSR that was not reflected in the findings in this chapter.

Considering these studies, the results presented here suggest that OSR genotypic diversity upon root and rhizosphere soil microbiomes is genotype and context dependent. Taye et al. (2020) and Bazghaleh et al. (2020) utilise OSR lines which were selected based upon differing seed parameters including global origin, seed colour, lignin content, glucosinolate concentration and erucic acid concentration. This is in comparison to the OSR genotypes selected in this study which were chosen to represent extremes of root density and plant metabolite exudate profiles. In addition, no community level differentiation was observed in a study of herbicide resistant and susceptible genotypes of OSR fungal microbiomes (Floc'h et al., 2020). This suggests that the specific manifestation of genotypic differences may be involved in whether there is an influence upon microbial community structures. In addition, edaphic, geographical and climactic factors known to influence root and rhizosphere soil microbiomes have been shown to overwhelm cultivar influences in Maize, adding complexity to plant genotype – microbiome dynamics (Chen et al., 2017). Indeed, it has been observed that studies of model organisms in laboratory conditions often show host control of the microbiome to some extent with the relationship become less clear and more complex in environmentally complex field settings (Tabrett and Horton, 2020).

2.5.2 Plant genotype and microbiome structure at the OTU level

Despite overlap of genotype microbiomes and the community level, significant differences between the genotypes were identified at the OTU level in terms of OTU fidelity and co-occurrence patterns within networks. Indicator species analysis identified significantly fidelitous bacterial, fungal and protist OTUs for each genotype.

Of particular interest, were the top indicator species of the fungal rhizosphere soil and root microbiomes of each genotype. These fungal OTUs belonged to a range of potential pathogenic and beneficial taxa. These included an OTU belonging to the Tetracladium Genus which has recently been positively correlated with yield in OSR across the UK (Hilton et al., 2021), an OTU belonging to the Fusarium Genus which are implicated as major plant pathogens and potentially beneficial endophytes in the Brassicacae (Card et al., 2015, Ma et al., 2013) and an OTU belonging to the pathogenic Phoma Genus (Bennett et al., 2018). In addition to these fungal indicator taxa, a top indicator OTU of the Canard protist root community was a member of the Pythium family of pathogenic protists (Martin and Loper, 1999). These results indicate that despite genotype microbiomes being largely similar in composition at the community level, specific OTUs within the shared communities may be more likely to associated with specific genotypes over others.

Inter-taxon co-occurrence networks presented in this study identified distinct network structures between genotypes and represent the first inter-taxon co-occurrence networks presented for OSR microbiomes. Genotype networks were compositionally similar (majority of co-occurring taxa were shared between genotypes) but structurally distinct with differences in, connectivity, modularity, co-occurrence patterns and network node roles. Most network hub taxa identified in each genotype were found to occupy less connected network roles in other genotypes. This may suggest that despite compositional similarity, interactions between taxa and the functions they have in each genotype's microbiome may change. Indeed, the shift in network roles may be of significance since network hub taxa have been considered community 'keystones' upon which microbiome structure and resilience is linked (Banerjee et al., 2018). Variation in the number and taxonomic identity of these network hub taxa were observed between the genotypes in both root and rhizosphere soil networks. More network hub taxa were identified in rhizosphere soil networks compared to root networks and were mainly formed of fungal taxa. Most network hub taxa were assigned at lower levels of taxonomic resolution and included taxa assigned as 'Unidentified fungi' and 'Unidentified Ascomycota'. This limited predictions of functional capabilities that can be drawn from taxonomy and is an inherent limitation of amplicon sequencing based approaches compared to other 'omics' approaches such as shotgun metagenomics and transcriptomics (Baldrian, 2019). In addition, since

OSR yield, and biomass were not significantly correlated with any OTUs, it is difficult to hypothesise the functional capabilities of network hub taxa in relation to and microbial community functionality and plant health.

However, a small number of network hub taxa were assigned at the species level, allowing identification of 'taxa of interest' for further analysis in other 'omics' or cultivation-based studies. These taxa include F_OTU64 *Itersonilia pannonica* and F_OTU83 *Trichoderma crassum* from the canard rhizosphere soil network, F_OTU46 *Cladobotryum rubrobrunnescens* from the temple rhizosphere soil network and F_OTU13 *Exophiala equina* from the catana root network. *I. pannonica* has been identified as a "likely pathogen" using FunGuild analysis in a study of ash dieback disease (Griffiths et al., 2020, Nguyen et al., 2016b). *T. crassum* has mainly been discussed in literature in a descriptive sense focussing on its taxonomic relationship to other Trichoderma species and its morphology (Chaverri et al., 2003, Hoyos-Carvajal et al., 2009). However, it has previously been screened for biocontrol activity against the pathogenic taxa *Sclerotinia sclerotiorum*, but no significant biocontrol activity was found (Jones et al., 2014). *C. rubrobrunnescens* is particularly interesting given that it is a mycophilic fungus and a close relative of the causal agents of cobweb disease found in agriculturally cultivated mushrooms (Tamm and Põldmma, 2013). It has also been found to produce antibiotics and cyto-/phytotoxic compounds (Wagner et al., 1995, Wagner et al., 1998). Most research regarding the Exophiala genus to which *E. equina* belongs concerns these taxa being pathogenic agents in cold blooded aquatic animals and opportunistic pathogens in other animals (de Hoog et al., 2011). However, members of this genus are frequently isolated from natural environments including plant roots (Addy et al., 2005, Maciá-Vicente et al., 2016).Indeed, a close relative of *E. equina*, *Exophiala radicis* has been isolated from the roots of the OSR relative *Microthlaspi perfoliatum* (Maciá-Vincente et al., 2016).

The data presented here shows that distinct microbiomes in terms of OTU connectivity and relationships were formed in each genotype within the study. Together with indicator species analysis, which identified significantly fidelitous OTUs in each genotype, this shows the potential for genotypic influences beyond the selection of taxonomically distinct microbiomes at the community level.

2.5.3 OSR biomass and rhizosphere microbiome community diversity

Differences in the overall community structure (β - dissimilarity) of rhizosphere microbiomes significantly explained between 5.57 – 6.75 % of OSR shoot/ root biomass and yield variation in certain genotypes. The bacterial root community of Catana significantly explained yield and was the only significant explanatory factor connected with yield identified in this study. Indeed, no individual taxon at any taxonomic level was found to significantly correlate with yield or biomass positively or negatively. This meant that network modules and structures identified in inter-taxon co-occurrence networks could not be related to positive or negative biomass trends, something which was observed in Hilton et al., (2021)'s investigation of the UK OSR rhizosphere microbiome. The lack of yield and biomass correlated OTUs is at odds with previous investigations which have identified a number of taxa positively and negatively correlated with biomass and yield in OSR (Alström, 2001, Hilton et al., 2013, Hilton et al., 2021, Kloepper et al., 2004, Lay et al., 2018a, Lay et al., 2018b). Certain yield correlated taxa identified in these studies were found in rhizosphere microbiomes of the genotypes assessed in this study including a Tetracladium species and *O. brassicae* (Hilton et al., 2013, Hilton et al., 2021). This indicates that other factors may contribute to and supersede the rhizosphere microbiomes contribution to biomass or the ability of OSR genotypes to cultivate beneficial or deleterious microbiomes at this local level.

Such influences already known to impact the microbiome and plant biomass include; edaphic factors (Schlatter et al., 2019), meteorological conditions (Brown et al., 2019, He et al., 2017), plant developmental stage (Rybakova et al., 2017) and agronomic practices (Berry and Spink, 2006, Hilton et al., 2013, Lay et al., 2018a, Rathore et al., 2017). Agronomic practices such as continuous cultivation are particularly important in OSR, as they associated with the formation of a deleterious rhizosphere microbiome and yield penalties (Knight et al., 2012, Hilton et al., 2013, Hilton et al., 2021). Since the field site utilised in our study had no history of OSR cultivation, this will have impacted the resident soil microbiome from which the OSR genotypes selected from. Therefore, it is unknown whether these genotypes cultivate differential rhizosphere microbiomes with yield boosting or protecting effects in response to continuous cultivation of OSR.

2.5.4 Directions for future research

The research presented here indicates that crop genotypes may influence the formation of root and rhizosphere soil microbiomes under field conditions at the OTU level, potentially 'selecting' from a similar microbial community. Different inter-taxon network structures and hub taxa were identified between genotypes and certain OTUs showed significant fidelity to different genotypes. In addition, this study also shows a relationship between microbial community variation and crop biomass and yield outcomes in certain genotype microbiomes in a real-world setting. However, there are limitations to the experimental design and techniques employed in this study which provide directions for future research.

Firstly, an inherent limitation of amplicon sequencing analysis is the inability to accurately observe and analyse functional processes occurring in the microbiomes it documents (Baldrian, 2019, Shakya et al., 2019). This provides challenges to functional interpretation and hypotheses building from data gained from inter-taxon co-occurrence networks. This is especially true in the absence of biomass/ yield correlated taxa such as the case in this study. By comparison, other investigations utilising network analysis have examined microbiomes between plants infected with pathogenic nematodes (Zhao et al., 2017), low and ultra-high yielding crop growth regions(Zhong et al., 2020) and at a landscape level (Hilton et al., 2021). For example, Hilton et al., (2021) constructed inter-taxon co-occurrence networks including yield and biomass correlated taxa at the landscape level, allowing the identification of potential 'keystone' taxa for further investigation. For example, the pathogenic taxa *O. brassicae* known to impact OSR yields (Hilton et al., 2013) was found to negatively correlate with yield in co-occurrence networks. This enabled identification of taxa connected to *O. brassicae* in these networks as taxa of interest. This included a protist OTU related to the Olpidiopsidales and Haliphtorales clades which was also found to significantly co-occur with the pathogenic fungal taxa *P. brassicae* and significantly co-exclude with the potentially beneficial endophyte *T. maxilleforme*. Therefore, further analysis including cultivation of these taxa to confirm these network relationships is possible and has previously been achieved in rice paddy rhizosphere bacterial microbiomes (Wang et al., 2017).

As discussed above, without such background knowledge and results to contextualise inter-taxon co-occurrence networks it can be difficult to hypothesise functional implications based on taxonomy alone. Coupling amplicon-based analysis with functional analyses like transcriptomics could more clearly reveal whether functional shifts occurred between genotype microbiomes and provide further context for co-occurrence networks and other OTU level analyses such as indicator species analysis (Shakya et al., 2019, Baldrian, 2019). This could strengthen the case for further investigating certain 'keystone' or network hub taxa beyond inference based on taxonomy as presented in this study.

Altering analysis parameters and experimental designs could also provide a better or new understanding of genotype – microbiome dynamics as presented in this study. Regarding analysis parameters, altering OTU inclusion criteria for network analysis, in this case being in the top 25 % most abundant taxa, could enable examination of the role of rare taxa in network structures and connections. The rare biosphere is of increasing interest due to the ability of rare taxa to influence microbial community structures and biogeochemical cycles (Jousset et al., 2017, Sogin et al., 2006). Therefore, inclusion of rare taxa can be seen as particularly important in further investigations, especially in the light of research which identified rare bacterial taxa as the main 'keystone' highly connected taxa in co-occurrence networks for salt marsh ecosystems (Du et al., 2020). Du et al., (2020) also document divergent assembly processes between abundant and rare taxa. This may indicate that rare and abundant taxa respond differently to different selective pressures and as such may respond differently to host genotype diversity. Therefore, inclusion of these rare taxa may be particularly important when considering further investigations based on the research presented here given that the genotypes assessed in this study exert influence at the OTU level.

In addition to the rare biosphere, further examination of "microbial dark matter" or the uncharacterised microbial taxa which comprise most of the microbial life on earth is of interest (Lloyd et al., 2018). This is especially true in the light of research which employed network analysis to understand the potential ecological roles of microbial dark matter (Zamkovaya et al., 2021). Zamkovaya et al., (2021) identified microbial dark matter taxa as key components of microbial network structures and identified many dark matter hub taxa. The research presented in this study also

identified several dark matter taxa occupying network hub or keystone taxa positions in individual genotype networks including taxa unidentified to the Phylum level. Together this study and the research presented by Zamkovaya et al., (2021) highlights the need for further investigation of microbial dark matter to elucidate their ecological roles and functionality.

In addition to incorporating new analysis techniques and altering analysis parameters, reconfiguring the experimental design employed in this study could also enable new insights into plant genotype-microbiome assembly dynamics. Changes to experimental design could include examination of rhizosphere microbiome structure across a temporal scale in each genotype and may help elucidate whether genotypes exert differential selective pressures on their resident microbiome at different plant growth stages. Indeed, such interplay between host genotype and plant age has been shown to effect changes in plant exudation profiles and subsequently the formation of genotype specific bacterial microbiomes (Micallef et al., 2009). In addition, extending examination of genotype microbiomes across multiple growing seasons and years may elucidate the ability of genotypes to select a genotype specific microbial assembly in the long term. This approach may be particularly prudent as agricultural rotation practices such as continuous cultivation are significantly associated with yield penalties and the development of deleterious microbiota in OSR (Hilton et al., 2013, Hilton et al., 2021, Knight et al., 2012). As the genotypes in this study were found to have significant indicator taxa and distinct co-occurrence networks, genotypic influences upon the rhizosphere may manifest in the suppression of deleterious taxa accumulating over many seasons instead of wholesale re-assembly of the microbiome at the community level. In addition to strategies centring around the genotypes investigated in this study, future investigations could include assessments of OSR genotypes with different phenotypic manifestations such as genotypes with resistance to the pathogenic protist *P. brassicae* or tolerances to abiotic stress factors.

3 Culture-dependent analysis of the effect of the OSR mycobiome on plant growth

3.1 Introduction

Rhizosphere microbiomes are key determinants of plant health (Berendsen et al., 2012). As such, research focusing on manipulating or 'engineering' microbiomes is gaining increasing interest as an agricultural tool (Foo et al., 2017, Trivedi et al., 2017). Rhizosphere microbiome engineering aims to develop beneficial rhizosphere microbiomes to confer traits such as improved disease resistance, abiotic stress tolerance and importantly, increased yields in crops. It is already known that certain taxa within these microbiomes such as mycorrhizal fungi and endophytic microbes can confer such properties (Chen et al., 2018, Yu et al., 2019). However, a systematic understanding of how the microbiota assembles and functions as a whole in relation to the plant host is not fully developed. This is a key knowledge gap in the understanding of the rhizosphere microbiome and a major roadblock to the development of functional microbiome manipulation as an agricultural tool.

Advancements in rhizosphere microbiome research, such as amplicon sequencing of taxonomic reference genes (i.e. 16S, 18S and the ITS gene regions) has allowed the exploration and documentation of microbiome assembly and richness to depths not possible through culture based studies (Knief, 2014). Such data provides information of community membership, diversity, and correlative relationships between taxa and metadata. There are also bioinformatic tools available such as FunGuild and PICRUSt, which can be used to infer trophic modes and functions of taxa using taxonomic reference gene sequences (Langille et al., 2013, Nguyen et al., 2016b). Other sequencing techniques such as shotgun metagenomics can provide an alternative approach to the analysis of PCR amplified taxonomic reference genes. In this technique, sequencing of the total DNA content of environmental samples is carried out (Lucaciu et al., 2019, Sharpton, 2014). As analysis is not restricted to sequencing one genome locus this enables functional characterization of reads, providing a more accurate assessment of functionality (Langille et al., 2013, Nguyen

et al., 2016b, Sharpton, 2014). This gives the chance to gain information on who is in the microbiome and what they may be doing from the same analysis.

Despite the progress made in sequencing techniques, definitive relationships between taxa and their function in relation to plant health, cannot be conclusively drawn from sequencing data alone. Functional inference based on taxonomy is limited by the constraints of tools such as FunGuild and PICRUSt. These tools rely on functional prediction based on taxonomic similarity to representative isolates. Functional capabilities of these references isolates are based on the collective research knowledge of their functional potential (FunGuild) and/or their full genome sequence (PICRUSt) (Langille et al., 2013, Nguyen et al., 2016b). Such information is available for taxa which are well-defined pathogenic or beneficial species, however, many taxa documented in sequencing profiles have little to no functional information attributed to them. This is especially true of fungi which have large genomes, and a smaller number of fully sequenced genomes in comparison to prokaryotes (Hibbett et al., 2013). In addition, different strains of the same microbial species may have significant differences in functional capabilities due to the presence or absence of certain functional genes within accessory genomes (McCarthy and Fitzpatrick, 2019). For example, different strains of the bacterial species *Bradyrhizobium japonicum* may or not be able to form symbioses with legumes due to the presence or absence of nodulation and nitrogen fixating genes in chromosomal "symbioses islands" (Sachs et al., 2011). This information may be gathered from alternative methods such as shotgun metagenomics. However, genome assembly coverages can vary for each constituent member of a microbiome, with low abundance genomes potentially fragmenting and strain level differences causing branching in assembled genomes (Sharpton, 2014). This can increase the difficulty of assigning functionally annotated genes to specific members of the microbiome. In addition, functional assignment of genes is based on similarity to protein reference databases which can have phylogenetic biases (i.e. some taxa are more thoroughly and/or accurately annotated than others) and protein families with no known function (Sharpton, 2014). Functional capabilities may also be situationally specific, and the presence or absence of certain genes may not accurately describe the specific function of microbiota in a certain system e.g. functional genes may not be being actively expressed (Sharpton, 2014).

Therefore, complementing sequencing based analyses with isolation and cultivation of axenic cultures is essential for carrying out plant-microbe interaction studies and full genome sequencing to provide information on taxa functional capabilities and potential. Conducting such research is especially important for analysing the functional capabilities and ecological relevance of rhizosphere fungal communities (Peay, 2014). Indeed, our understanding of the relevance of rhizosphere fungal communities to plant health and microbiome structure is less advanced than that of bacteria (Pozo et al., 2021). Most functional research has focussed upon plants with known mycorrhizal associations or the plant pathogenic processes of certain fungal taxa. This is not surprising given the importance of mycorrhizal associations and pathogenic taxa to plant health (Chen et al., 2018). However, this leaves key knowledge gaps in our functional understanding of non-mycorrhizal interactions and of rhizosphere fungi as a whole.

A combined amplicon sequencing – culture approach is exemplified in Hilton et al. (2013) which sought to understand the relationship between agricultural rotation practices, yield decline and the rhizosphere microbiome. Continually cultivated OSR rhizosphere microbiomes were found to have a significantly higher abundance of the fungal species *Olpidium brassicae* and *Pyrenochaeta lycopersici* compared to rotated and virgin OSR. Isolates of these fungi were then screened against OSR plants in a laboratory setting, identifying negative impacts on plant biomass such as reducing; top growth/root biomass, branching, pod production and seed production for *O. brassicae* and, reduced seed number, quality, and delays in flowing time for *P. lycopersici*. This combined approach allowed the identification of potentially significant fungal taxa using amplicon sequencing methods, selective isolation of target fungi, and subsequent demonstration of a causal relationships between fungi and plant growth, which explained effects seen in the field. Therefore, this combined approach is key to the identification and study of potentially significant fungal taxa which are associated with crop species.

In OSR, several studies have been presented which have documented rhizosphere mycobiome assembly (**Table 3.1**). However, functionality of the rhizosphere fungal microbiome has not been extensively researched beyond analysis of the causative agents of major OSR diseases and a small body of research on

Table 3.1 List of studies which have examined OSR mycobiome assembly

Reference	Study type	Community analysis method(s)	Study location/ inoculum source
(Hilton et al., 2021)	Field	Nextgen sequencing (MiSeq™) Target region: ITS	UK wide OSR cultivation region
(Floc'h et al., 2020)	Field	Nextgen sequencing (MiSeq™) Target region: ITS	Alberta & Saskatchewan, Canada
(Bazghaleh et al., 2020)	Field	Nextgen sequencing (MiSeq™) Target region: ITS	Saskatchewan, Canada
(Schlatter et al., 2019)	Field	Nextgen Sequencing (MiSeq™) Target Region: ITS	Washington State, USA
(Lay et al., 2018a)	Field	Nextgen Sequencing (MiSeq™) Target Region: ITS	Alberta & Manitoba, Canada
(Hale, 2017)	Field	Nextgen Sequencing (MiSeq™) Target Region: ITS	Hertfordshire, UK
(Zhao et al., 2017)	Field	qPCR & Nextgen Sequencing (HiSeq™) Target Regions: ITS	Hubei, China
(Gkarmiria et al., 2017)	Laboratory Microcosm	DNA SIP & 454 Pyrosequencing Target Region: ITS	Ultuna, Sweden
(Hilton et al., 2013)	Field & Laboratory microcosm	RT-PCR & TRLFP ITS	Warwickshire, UK

beneficial taxa. Regarding the major fungal pathogens of OSR (*S. sclerotiorum*, *L. maculans*, *L. biglobosa*, *V. longisporum* and *R. solani*), functional analysis has identified significant impacts on OSR biomass and achieved yields due to invasion of taxa in OSR tissues and presence in rhizosphere soils (AHDB, 2021, Zheng et al., 2020). By comparison, research concerning beneficial fungi in OSR has been limited and may be due in part to the fact that OSR is considered a non-mycorrhizal plant species (Cosme et al., 2018). However, recent research which examines the blurred lines between mycorrhizal and non-mycorrhizal plant species has stressed the significant beneficial impacts that root associated fungi can have upon non-mycorrhizal plants such as OSR (Cosme et al., 2018), and evidence suggests that presence of some fungal root endophytes may be positively associated with OSR health (Card et al., 2015). These findings give merit to the exploration of fungi as beneficial associates to non-mycorrhizal species. Indeed, fungal isolates have been documented to show plant beneficial traits for OSR in a review of OSR endophytes (Card et al., 2015). The beneficial fungi identified in these studies had a range of beneficial traits including; plant growth promotion (*Alternaria alternata*, *Fusarium tricinctum* and *L. biglobosa*), antifungal activity (*Chaetomium globosum*, *Fusarium oxysporum* and *L. biglobosa*), disease severity reduction (*Aspergillus flavipes*), pest larval inhibition (*Metarhizium anisopliae)* and assistance of beneficial colonisation of mycorrhiza into OSR roots (*Trichoderma harzianum)* (Batta, 2013, Poveda et al., 2019, Zhang et al., 2014). Given that a number of these taxa are known plant pathogens, specifically a major pathogen of OSR in the case of *L. biglobosa*, and that a mycorrhiza conferred beneficial traits in a non-mycorrhizal plant, this suggests that fungal plant interactions in this crop species are complex and nuanced.

As previously stated, studies which combine amplicon sequencing techniques with culture screening are necessary to evaluate the impact of rhizosphere associated fungi and their function in relation to the plant host (Peay, 2014). This is due to the limitations of each methodology when employed alone. Recently, a landscape level analysis of the OSR rhizosphere microbiome has been presented (Hilton et al., 2021). Therefore, it is now possible and pertinent to use such a dataset in combination with a culture-based study to evaluate the ecology and functionality of OSR rhizosphere mycobiomes.

3.2 Aims

In this chapter, it was hypothesised that using a combination of cultivation based and sequencing based investigations would help isolate and select ecologically and functionally relevant OSR associated fungi for further investigation .To these ends, a culture collection of OSR rhizosphere associated fungi was isolated and curated using the landscape level OSR microbiome dataset presented by Hilton et al., (2021) to:

1. Isolate a collection of fungal isolates from OSR roots and rhizosphere soil which is representative of the UK OSR rhizosphere mycobiome.

2. Select ecologically relevant isolates which were either; widely distributed, highly abundant and/or significantly correlated with OSR biomass for prioritisation in co-incubation experiments.

3. Carry out co-incubation experiments using isolates of interest on OSR seedlings to screen for functionally beneficial or deleterious fungal isolates.
4. .

3.3 Methods

3.3.1 Fungal culture collection creation

3.3.1.1 Collection of plant materials for fungal isolation

OSR plants used for isolation of root and rhizosphere fungal taxa were harvested from Hunts Mill Field, University of Warwick's Wellesbourne campus, UK, in April 2017 at the OSR bud break development stage, immediately prior to flowering **(Figure 3.1)**. The soil is a sandy loam of the Wick series with a composition of 73% sand, 12% silt, and 14% clay (Whitfield, 1974). Samples were taken using a W-shaped sampling pattern starting at least 25 m into the field to avoid edge effects. One 'W' transect was marked out with canes 10 m apart. Plants closest to the canes were harvested by holding the base of the stem at the soil interface with a gloved hand and gently pulling with the aid of a sterilised trowel. Excess adhering soil was removed by

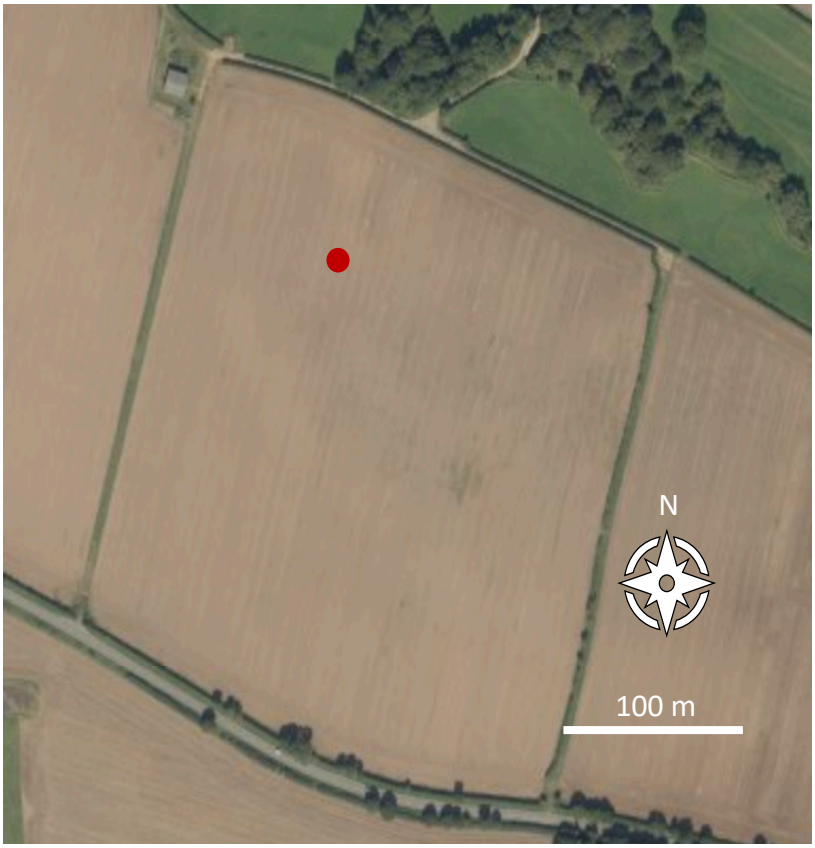

Figure 3.1 Isolation site of OSR associated fungal isolate collection. *Centre of W transect indicated by red point. Coordinates of isolation site are 52°11'59.0"N 1°36'41.6"W situated in Hunts Mill Field, University of Warwick Wellesbourne campus, UK. Map obtained from Ordnance Survey online database March 2021 (Contains OS data © Crown copyright and database rights 2021).*

tapping the plant against the trowel. Two plants from each 'W' cane were pooled resulting in a composite sample for isolation of fungi. Plants were then stored in a plastic bag within a cool box for transport to the university within one hour. Plants were then subsequently stored at 4 °C and fully processed within 48 hours of harvesting. The root systems with closely adhering rhizosphere soil were collected by cutting at the root-stem interface with sterilised scissors and tweezers on a benchtop under a Bunsen flame before being transferred to sterile Falcon tubes. Subsequent work was carried out in a laminar flow cabinet with aseptic technique applied to all processes.

3.3.1.2 Rhizosphere soil and root tissue collection

Loosely adhering soil was removed from roots by gentle tapping with a 70 % ethanol sterilised trowel, leaving no more than 2 mm rhizosphere soil. Rhizosphere soil was collected by placing individual collected root system into a 50 ml falcon tube containing 25 ml of sterile deionised water (SDW) with vigorous shaking for 30 seconds. Root systems were then transferred to a fresh 50 ml falcon tube and the soil collection repeated. Once soil was collected root systems were transferred to a sterile beaker and stored at 4 °C until needed. Collected soil washes were then centrifuged at 5000 g for 20 minutes to pellet soil. Supernatant were then discarded, and soil pellet weights noted. Soil pellets were then stored at 4 °C until required for inoculation. After rhizosphere soil collection roots were harvested from tap roots of the washed root systems using sterilised scissors and tweezers. Harvested lateral roots were transferred to a sterilised beaker and cut into approx. 1-2 cm pieces using sterilised tweezers and scissors and thoroughly mixed. At this stage, half of the collected root tissue was reserved and termed 'washed' roots and half reserved for surface sterilisation treatment. Surface sterilisation was carried out by transferring whole root systems to a beaker containing 0.033 % sodium hypochlorite solution in SDW + one drop of tween 80 for 1 minute. Surface sterilised roots were then transferred to a beaker containing SDW for 1 minute to remove sterilisation solution and transferred to a dry sterile beaker and stored at 4 °C until required for inoculation. 100 µl of SDW pre and post root exposure and the sterilisation solution were then plated in triplicate onto PDA agar and incubated at 15 °C for one week to check sterility and success of surface sterilisation treatment. Microbial growth was identified on one of post root exposed SDW control plates, indicating that the surface sterilisation treatment was

unsuccessful, and fungi isolated from these tissues are not restricted to endosphere inhabiting fungi only.

3.3.1.3 Rhizosphere soil and root tissue inoculation preparation

For preparation of rhizosphere inoculation suspensions, the combined weight of first and second wash pellets were calculated and SDW added at a ratio of 0.1 g/ml to resuspend and pool both washes. After vortexing for 30 seconds, 1 ml of rhizosphere soil suspension from each plant was taken from the middle of the suspension using a wide bore pipette tip and pooled. This pooled rhizosphere wash sample was used for inoculation of media using a fivefold serial dilution in SDW from 0 to 10th dilution. For preparation of root inoculation suspensions and fragments, 'washed' and 'surface sterilised' roots were separated into two aliquots. Roots fragments were created by cutting processed roots into 5 mm sections using sterilised scissors and tweezers. Root fragments were then immediately inoculated onto growth media described in section 3.3.1.3. Root serial dilutions were created by weighing an aliquot of roots, grinding in a sterile disposable tissue grinder, and suspending in SDW in a ratio of 0.1 g fresh root to 1 ml SDW. This was then used for used for inoculation of media using a fivefold serial dilution from 0 to 10^{th} dilution.

3.3.1.4 Fungal growth media, inoculation, and isolation procedures

Potato Dextrose agar (Oxoid) (PDA agar), Rose Bengal Chloramphenicol agar (Oxoid) (RBC agar), Modified Melin-Norkrans C Medium (MMN) and 25 % Oilseed Rape agar (0.25 OSR agar) were utilised in this study (**Table 3.2**). Media was prepared as per manufacturer's instructions for PDA and RBC agar, as described by PhytoTechnology Laboratories® MMN agar composition for MMN agar and as described in (Hilton et al., 2013) for 0.25 OSR agar. All media was sterilised at 121 °C, 15 psi for 15 mins. 0.25 OSR was used to encourage the growth of OSR specialist taxa. MMN agar was used to encourage the growth of plant associated taxa including mutualistic taxa. RBC agar was used to slow the growth of fungal taxa and to allow the isolation of slower growing fungal taxa.

Serial dilutions of; rhizosphere soil wash, washed roots and surface sterilised roots from 0 to 10^{th} dilution of a fivefold serial dilution were plated onto each agar in triplicate (100 µl inoculum), spread with sterile spreaders and allowed to dry in a laminar flow cabinet. SDW used to wash off surface sterilising agents for surface

sterilised roots was plated in triplicate onto each agar to check the surface sterilisation procedure. Colony growth was noted on RBC agar inoculated with SDW used in surface sterilisation, indicating that sterilisation procedures were not successful. Once inoculate fully dried, plates were stacked, wrapped in clingfilm, and incubated at 15 °C for up to 6 weeks. For root fragment plating, individual root fragments were placed onto 5 ml 8 well plates filled with each agar and incubated at 15 °C for up to 6 weeks.

Isolation of fungi was carried out weekly from 5 days post inoculation to 6 weeks post inoculation. Fungi were isolated from each well or plate based on colony morphologies including, colour, shape, and hyphal growth with a maximum of three replicates of each morphology isolated from each well or plate. Isolates were sub-cultured onto the same media as isolated from until pure colonies were obtained. From these fungi were maintained on PDA agar for application in co-incubation studies and for production of mycelia for DNA extraction.

Table 3.2 Fungal & plant growth media recipes utilised in this study

isolation media	ingredients
Rose Bengal Chloramphenicol Agar	• RBC Agar powder (Oxoid) – 16 g • Chloramphenicol 35 mg/ml – 1.75 ml • De-ionised H_2O – up to 500 ml
Oilseed Rape Extract	• Washed and macerated Oilseed Rape Plants – 500 g • De-ionised H_2O – 500 ml
0.25 Oilseed Rape Extract Agar	• Oilseed Rape Extract – 125 ml • Technical Agar (Oxoid) – 6 g • Streptomycin 50 mg/ml – 1 ml • De-ionised H_2O – up to 500 ml

Modified Melin-Norkrans Medium	• Ammonium Phosphate, Monobasic – 92.35 mg • FeNaEDTA – 10 mg • Magnesium Sulphate – 17.9 mg • Potassium Phosphate, Monobasic – 250 mg • Sodium Chloride – 12.5 mg • Calcium Chloride, Anhydrous – 25 mg • Thiamine, HCl – 0.05 mg • Malt Extract – 15 g • Sucrose – 5 g • Agar – 3.5 g • Streptomycin 50 mg/ml – 1 ml • De-ionised H_2O – up to 500 ml
½ Hoagland's Basal Salt Solution	• 500 ml Hoagland's No.2 Basal Salt (Sigma-Aldrich, UK) suspended in 1 L SDW. • 500 ml SDW

3.3.1.5 Identification of isolates via sanger sequencing

DNA was extracted from fungal mycelia grown on PDA agar using a FastDNA™ kit for Soil (MP Biomedicals, Irvine, USA) with mechanical disruption of cells carried out using a FastPrep-24™ homogeniser (MP Biomedicals, Irvine, USA) for 2 x 30 seconds at a speed of 6 m s^{-1}. Isolated DNA was then quantified using an Implen NanoPhotometer® NP80 and diluted to between 1 – 10 ng/µl using molecular grade water for PCR. PCR was carried out using untagged ITS1 and ITS4 primers (White et al., 1990) for fungal ITS regions with the following programme 98 °C 30 s, (30 cycles of: 98 °C 10 s, 57 °C 15 s, 72 °C 20 s), 70 °C 5 min, cool to 4 °C. PCR product clean-up was carried out using Ampure PCR clean up beads. Samples were sent for sequencing using Eurofins PCR 96 well barcode plates as per instructions (Eurofins, Luxembourg). Returned forward and reverse sequences were aligned, and individually quality controlled using SeqManPro. Identification of isolates were assigned using NCBI BLAST specified for highly similar sequences. Identities were subsequently used for prediction of trophic mode/ guild using

FUNGuild (Nguyen et al., 2016b). FUNGuild result graphs were produced using the ggplot2 package (Wickham, 2016) with the following add-on packages; ggpubr (Kassambara, 2018), ggrepel (Kamil Slowikowski, 2019), ggalt (Bob Rudis, 2017), ggforce (Lin Pedersen, 2019), cowplot (O. Wilke, 2019) and ggfortify (Tang, 2016, Yuan Tang, 2016) .

3.3.2 Identification of ecologically relevant fungal collection isolates

Culture collection samples were aligned against OSR fungal sequences identified in the UK wide OSR rhizosphere microbiome dataset presented in (Hilton et al., 2021) in order to identify ecologically relevant isolates. Alignment was carried out using a NCBI BLAST search with local database set to the ITS OTU FASTA file from (Hilton et al., 2021). Culture collection isolates were matched with a specific OTU using a 98% identity cut-off value. The ITS OTU table from (Hilton et al., 2021) filtered by culture collection matched OTUs was then processed in R to identify whether the culture collection was representative of OSR rhizosphere mycobiomes, and to identify culture collection isolates which were ecologically relevant in terms of landscape distribution, abundance and/or significant correlation with OSR biomass. This filtered OTU table was processed in the R software environment using the phyloseq (McMurdie and Holmes, 2013) and vegan (Jari Oksanen 2019) microbial community and microbial ecology analysis packages in order to produce relative abundance graphs. Graphs were produced using the ggplot2 package (Wickham, 2016) with the following add-on packages; ggpubr (Kassambara, 2018), ggrepel (Kamil Slowikowski, 2019), ggalt (Bob Rudis, 2017), ggforce (Lin Pedersen, 2019), cowplot (O. Wilke, 2019) and ggfortify (Tang, 2016, Yuan Tang, 2016).

3.3.3 Co-incubation studies

3.3.3.1 Preparation of fungal inoculant

Fungal isolates were grown on sterile barley grain media to provide bulk mycelial biomass for co-incubation studies with OSR seedlings. Sterilised barley was prepared by soaking de-husked barley grain (Tesco, UK) in freshly boiled deionised water for 30 mins. Softened barley grain was then rinsed with 1 L of deionised water three times to remove leached starch from grains. Barley grains were then left to air

dry for 30 mins. 150 g of grain was then transferred to a 500 ml flip-top borosilicate glass jar, wrapped in aluminium foil, and autoclaved at 121 °C, 15psi for 20 mins. All subsequent steps were carried out in a laminar flow hood under sterile conditions unless otherwise stated. To provide inoculant for of barley media, fungal isolates were grown on PDA for two weeks with 5 x 5 mm cubes of growing edge mycelia inoculated into barley media jars. 50 ml of SDW was then added to jars and mycelia agar cubes were gently mixed into barley grain media with a sterile hockey stick spreader to inoculate all barley grains. An un-inoculated control was prepared by adding 50 ml of SDW to barley grain media. Inoculated and control barley grain media jars were then sealed with Parafilm (Sigma-Aldrich, UK) and transferred to a 30 °C incubator for a period of 4 weeks.

50 g of barley grain media which were to be used in co-incubation studies was aliquoted into sterile 50 ml falcon tubes for transfer to University of Warwick Phytobiology facility on day of experimental commencement. To calculate the CFU of fungal inoculants used in the study and to check sterility of control media, a 0.5 g aliquot of each barley grain media was homogenised in 5 ml of SDW using a sterile ceramic mortar and pestle. A 1 in 10 serial dilution from 0 to 3rd dilution was prepared from this homogenate with 50 µl of each dilution inoculated in triplicate onto PDA plates. Inoculated PDA plates were sealed with Parafilm (Sigma-Aldrich, UK) and incubated a 30 °C for up to 4 weeks. CFU plates were checked daily for growth of colonies with counts documented.

3.3.3.2 Experimental design

Fungi were co-inoculated with OSR to identify fungal isolates which had a significant effect on OSR biomass. Microcosm design (**Figure 3.2 A**) consisted of open pots with a sharp sand (University of Warwick Phytobiology facility, Coventry, UK) and absorbent clay granule (Anderco, Ireland) growth medium amended with a fungal inoculum, un-inoculated barley (control 1) and no amendment (control 2). Growth media was prepared by mixing sharp sand and absorbent clay granules in a 50:50 mix by volume. This mixture was then autoclaved at 131 °C, 15 psi for 20 mins and stored at 5 °C overnight for use the subsequent day. Inside a laminar flow hood, this media was then inoculated with 3 % (by weight) fungal inoculant, un-inoculated barley or no inoculant. ½ Hoagland's solution (Sigma-Aldrich, UK) (**Table 3.2**) to 1/3 maximum water holding capacity (max water holding capacity = 0.398 gH_2O/g

sand/clay) of the sand-clay mix was then added and the sand-clay mixture massaged gently to thoroughly incorporate inoculant. This mixture was then added to fill five 7 cm square plant pots with pots placed onto sterile petri dishes to provide a stable base. Five replicate pots were prepared for fungal inoculated barley amendment, un-inoculated barley amendment (control 1) and no amendment (control 2).

Five OSR (*B. napus* var Compass: UK Vegetable Genebank, Wellesbourne Campus, University of Warwick, Warwickshire, UK) seeds were then sown equidistant onto the sand clay mixture and a thin layer of autoclaved perlite placed on top of seeds. Individual pots were then sealed in a 0.02 µm pore SunBag© (Sigma-Aldrich, UK) to prevent cross contamination and ordered in a randomised design. This randomized design was achieved by assigning pot numbers and randomizing order using a computerized list randomizer (**Figure 3.2 B**).

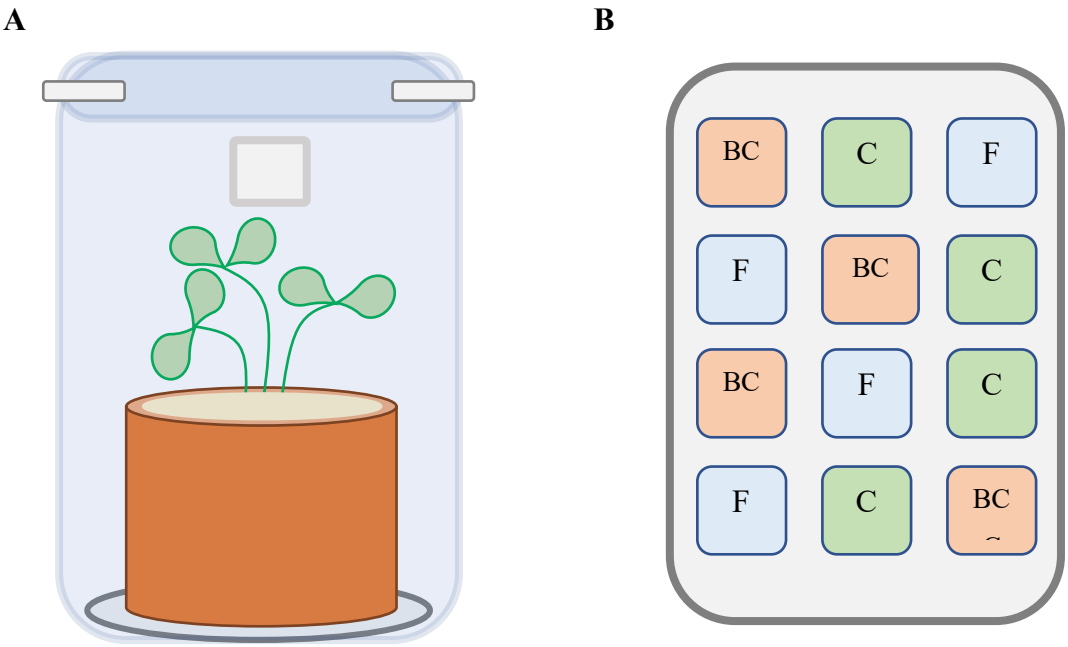

Figure 3.2 Plant-fungi screen experimental design. *(**A**) Diagram of assembled experimental design (**B**) Example of randomised design layout for experimental conditions (BC = amended with un-inoculated barley, F = amended with barley inoculated live fungus, C = no amendment control).*

Pots were maintained in controlled glasshouse conditions (12 hr light, 21 °C day, 18 °C night) for four weeks at the University of Warwick Phytobiology facility, Coventry, UK. Plants were then maintained once per week by supplementing with 7 ml SDW to maintain 1/3 max water holding capacity in pots. Plants were thinned to

one plant per pot at the week two timepoint. After four weeks, plants were harvested by gently separating root systems from potting media. Root systems were then collected by separating at the root stem interface with scissors and washed twice by shaking in water for 30 seconds in a 50 ml universal tube. Root systems were then blotted dry and weighed along with shoot systems for record of fresh weight. Leaf number was also recorded. Individual plant root and shoot systems were then placed into paper bags and dried for four days at 70 °C in a drying oven. Dried plant materials were then weighed to record root and shoot dry weight.

3.3.3.3 Statistical analysis

Statistical differences in plant biomass data between treatments were assessed using the Kruskal-Wallis test using the base R package with Dunn's Test post hoc analysis from the FSA R package, with Bonferroni multiple comparisons correction (Ogle et al., 2021, R Core Team, 2018, R Core Team, 2013).

3.4 Results

3.4.1 Isolation and taxonomic assignment of culture collection isolates

A total of 101 fungal isolates were obtained from the roots and rhizosphere of the ten OSR plants collected for this study. Of these 101 isolates, sufficient DNA was extracted from 74 isolates which were successfully sequenced using Sanger sequencing with taxonomic identities assigned using the BLAST NCBI database (**Table 3.3**). Together, 30 isolates were isolated from rhizosphere soil (40.5 %), 4 from homogenised root tissue dilutions (5.4 %) and 40 from root fragments (54.1 %) (**Figure 3.3**). Regarding isolation media, 22 isolates were isolated on MMN agar, 30 on OSR agar and 22 on RBC agar (**Figure 3.3**). The proportion of isolates from each tissue differed between agar with most isolates from MMN and OSR agar coming from root fragments (17 isolates 77.3 % and 18 isolates 60% respectively) and from rhizosphere soil in RBC agar isolates (16 isolates 72.7 %) (**Figure 3.3**).

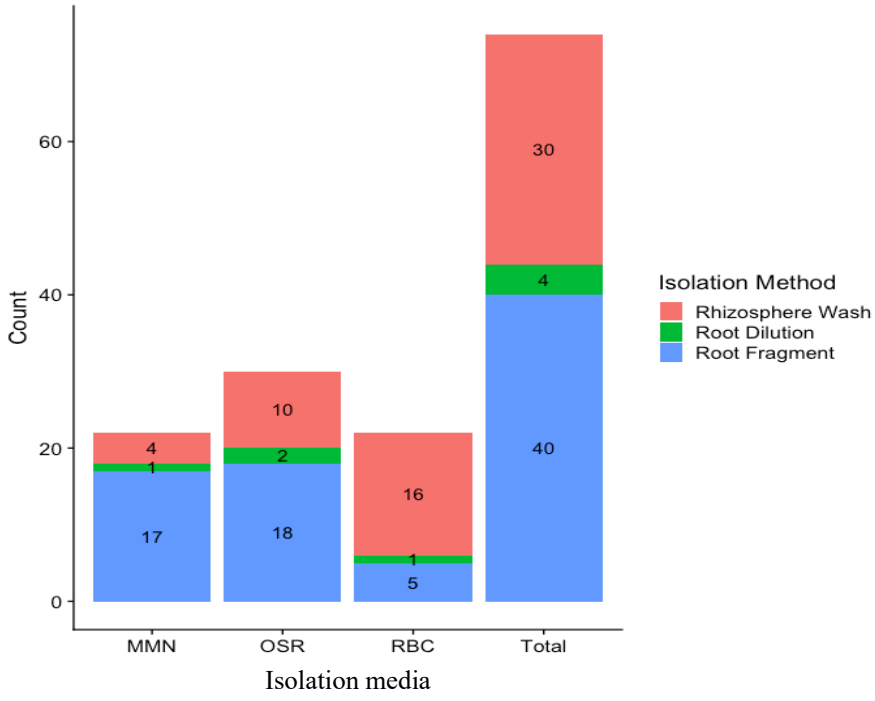

Figure 3.3 Stacked bar plots of sequenced culture collection isolates by isolation method and culture medium. *Counts of isolates by culture medium and total collection. MMN = Modified Melin Norkrans Agar, OSR = Oilseed Rape Extract Agar, RBC = Rose Bengal Chloramphenicol Agar, Total = All isolation media combined. Numbers are representative of Isolation method count. N = 74.*

Table 3.3 Culture collection isolate taxonomic ID as assigned by NCBI BLAST.

isolate id	matched taxonomic id	query length (bp)	query cover	identity	accession no.
RFMMN1	*Penicillum sp.*	509	99%	99.41%	MT975231.1
RFMMN10	*Mucor sp.*	624	100%	99.84%	KC888989.1
RFMMN11	*Harzia valata*	523	97%	99.02%	KY623411.1
RFMMN12	*Penicillium aurantiogriseum*	712	81%	99.45%	KY552626.1
RFMMN13	*Dactylionectria hordeicola*	565	96%	99.45%	MF440368.1
RFMMN14	*Fusarium culmorum*	510	99%	100.00%	MT032713.1
RFMMN2	*Phomopsis sp.*	611	93%	99.47%	MT278345.1
RFMMN3.1	*Trichoderma sp.*	643	93%	100.00%	MK870188.1
RFMMN3.2	*Trichoderma sp.*	559	99%	99.64%	MN944534.1
RFMMN3.3	*Trichoderma sp.*	574	100%	100.00%	MH284004.1
RFMMN3.4	*Trichoderma sp.*	497	100%	100.00%	MF565407.1
RFMMN4	*Fusarium asiaticum*	568	96%	100.00%	MK791240.1
RFMMN5	*Fusarium oxysporum*	480	100%	100.00%	MT530269.1
RFMMN7	*Mucor circinelloides*	651	96%	100.00%	MK087755.1
RFMMN8	*Dactylionectria hordeicola*	506	99%	99.41%	MF440368.1
RFMMN9	*Penicillum sp.*	556	98%	100.00%	GU566263.1
RFMMNXA	*Trichoderma viride*	597	93%	100.00%	MK290390.1
RFOSR1	*Geomyces pannorum*	586	96%	100.00%	AJ509866.1
RFOSR11	Uncultured Michrodochium	546	99%	99.63%	KY430557.1
RFOSR17	*Fusarium tricinctum*	602	93%	100.00%	MH681150.1
RFOSR18	Uncultured Michrodochium	593	93%	100.00%	KY430557.1
RFOSR19	Uncultured Michrodochium	521	99%	99.42%	KY430557.1
RFOSR2	*Rhizopus stolonifer*	593	99%	99.83%	DQ767605.1

RFOSR20	*Mortierella elongata*	629	93%	99.49%	MT453294.1
RFOSR3	*Neonectria sp.*	513	98%	99.80%	KC218449.1
RFOSR4	*Fusarium sp.*	487	100%	100.00%	KY318498.1
RFOSR5	Uncultured Dendryphion	575	94%	99.82%	HG937148.1
RFOSR7	*Phomopsis sp.*	532	99%	99.44%	HM771001.1
RFOSR8	*Tetracladium maxilliforme*	526	100%	99.81%	MK353128.1
RFOSR9	*Leptosphaeria biglobosa brassicae*	576	99%	99.83%	MH863069.1
RFOSRAA1	*Dactylonectria hordeicola*	510	98%	99.40%	MF440368.1
RFOSRXC	*Leptosphaeria biglobosa brassicae*	571	100%	99.82%	MH863069.1
RFRBC1	*Dactylonectria hordeicola*	518	98%	99.22%	MF440368.1
RFRBC3	*Trametes sp.*	548	100%	99.82%	MK509800.1
RFRBC4	Uncultured Mortierella	681	94%	99.84%	HG935763.1
RFRBC6	Uncultured Mortierella	578	98%	97.03%	GU327521.1
RFRBC7	*Fusarium sp.*	543	100%	100.00%	KJ935011.1
RHZDI1MMNX	*Trichoderma koningiopsis*	571	99%	99.65%	KU645324.1
RHZDI1RBC1	*Trichoderma tomentosum*	508	99%	99.60%	KX343120.1
RHZDI2MMN1	*Mucor hiemalis*	677	94%	100.00%	MT084004.1
RHZDI2MMN5	*Juxtiphoma eupyrena*	463	99%	100.00%	MK907745.1
RHZDI2OSR1	*Neonectria sp.*	508	99%	99.80%	KC218449.1
RHZDI2OSR2	*Harzia valata*	505	99%	100.00%	KY623411.1
RHZDI2RBC2.1	*Cystofilobasidium macerans*	631	93%	99.49%	MN128837.1
RHZDI2RBC2.2	*Cystofilobasidium macerans*	661	95%	99.68%	MN128837.1
RHZDI2RBC4	Uncultured Zygomycete	651	98%	100.00%	EU490047.1
RHZDI2RBCX1	Uncultured Cryptococcus	597	94%	100.00%	KY430574.1
RHZDI2XDRBC	Uncultured Trichoderma	599	100%	99.50%	HG937081.1
RHZDI3OSR3.1	Uncultured Mortierella	611	100%	99.67%	HG935763.1

RHZDI3OSR3.2	*Mucor hiemalis*	688	94%	100.00%	MF615076.1
RHZDI3OSRAA1	Uncultured psiloglonium	579	93%	100.00%	HG935441.1
RHZDI3OSRAA2	*Cladosporium sp.*	581	93%	100.00%	KX721645.1
RHZDI3OSRXB	*Penicilium canescens*	540	99%	99.63%	MH865756.1
RHZDI3RBC1	*Leptosphaeria biglobosa brassicae*	514	100%	99.81%	MN275884.1
RHZDI3RBC2	*Leptosphaeria biglobosa brassicae*	637	92%	100.00%	FO905468.1
RHZDI3RBC3	*Cryptococcus sp. HB 982*	599	92%	100.00%	FN430734.1
RHZDI4OSR1	*Cladosporium cladosporoides*	576	95%	99.64%	KU182497.1
RHZDI4OSR2	*Leptosphaeria biglobosa brassicae*	553	98%	99.63%	FO905468.1
RHZDI4RBC1	*Leptosphaeria biglobosa brassicae*	553	100%	99.82%	FO905468.1
RHZDI4RBC3	*Rhizopus stolonifer*	607	99%	99.67%	DQ767605.1
RHZDI4XAMMN	*Cladosporium cladosporoides*	583	93%	99.82%	MF077224.1
RHZDIRBC4	*Mucor hiemalis*	568	99%	95.93%	MT366055.1
RHZOSRX1	*Leptosphaeria biglobosa brassicae*	629	93%	99.83%	MH861366.1
RHZRBCX1	*Cladosporium anthropophilum*	539	94%	100.00%	MN515363.1
RHZRBCX2	*Cladosporium cf. subuliforme*	523	100%	100.00%	KX788171.1
RHZRBCX4	*Cladosporium cf. subuliforme*	523	100%	100.00%	KX788171.1
SRFOSR2	*Dendryphion nanum*	512	98%	99.61%	LT821405.1
SRFOSR3	*Phomopsis sp.*	606	93%	99.65%	HM771001.1
WRFOSRA1	*Cladosporium sp.*	559	96%	100.00%	KX721645.1
WRODI1OSR1	*Pseudogymnoascus pannorum*	557	93%	100.00%	KF986442.1
WRODI2MMN1	*Geomyces pannorum*	538	97%	99.24%	AJ509868.1
WRODI2OSR2	*Trichoderma sp.*	626	94%	100.00%	MK870382.1
WRODI2RBC1	*Trichoderma sp.*	638	94%	100.00%	MK871236.1

3.4.2 Inference of taxa ecological function using FunGuild

Function analysis based on isolate taxonomic ID was carried out using the FunGuild fungal guild assignment tool. Functional guilds were successfully assigned to each isolate (**Table 3.4**). 41 isolates were assigned to a functional guild with a single functional description i.e., Plant Pathogen, Soil Saprotroph, Undefined Saprotroph and Wood Saprotroph (**Table 3.4 & Figure 3.4**). Of these, Undefined Saprotrophs were the most represented guild of the collection with 26 isolates followed by Plant Pathogens with 11 isolates. Most isolates assigned to a functional guild with a single description were assigned at a 'probable' confidence level (21 isolates), followed by 19 'possible' and 1 'highly probable' confidence assignments (**Table 3.4**). Isolates were also assigned to functional guilds with multiple functional descriptions indicating uncertainty of assignment or fungal taxa with multiple diverse trophic modes in different ecological niches. In total, 33 isolates were assigned to these multi-function guilds (**Table 3.4 & Figure 3.4**). These guilds were different combinations of symbiotic (endophyte), pathogenic (animal pathogen, plant pathogen, parasite) and saprotrophic (wood saprotroph, soil saprotroph, litter saprotroph) functional guilds. Confidence level of assignments were also predominantly assigned as 'possible' (27 isolates) with only 6 isolates assigned at a 'probable' confidence level. This indicated that functional guild assignment of these isolates was not assigned to a high degree of confidence as dictated by FunGuild analysis.

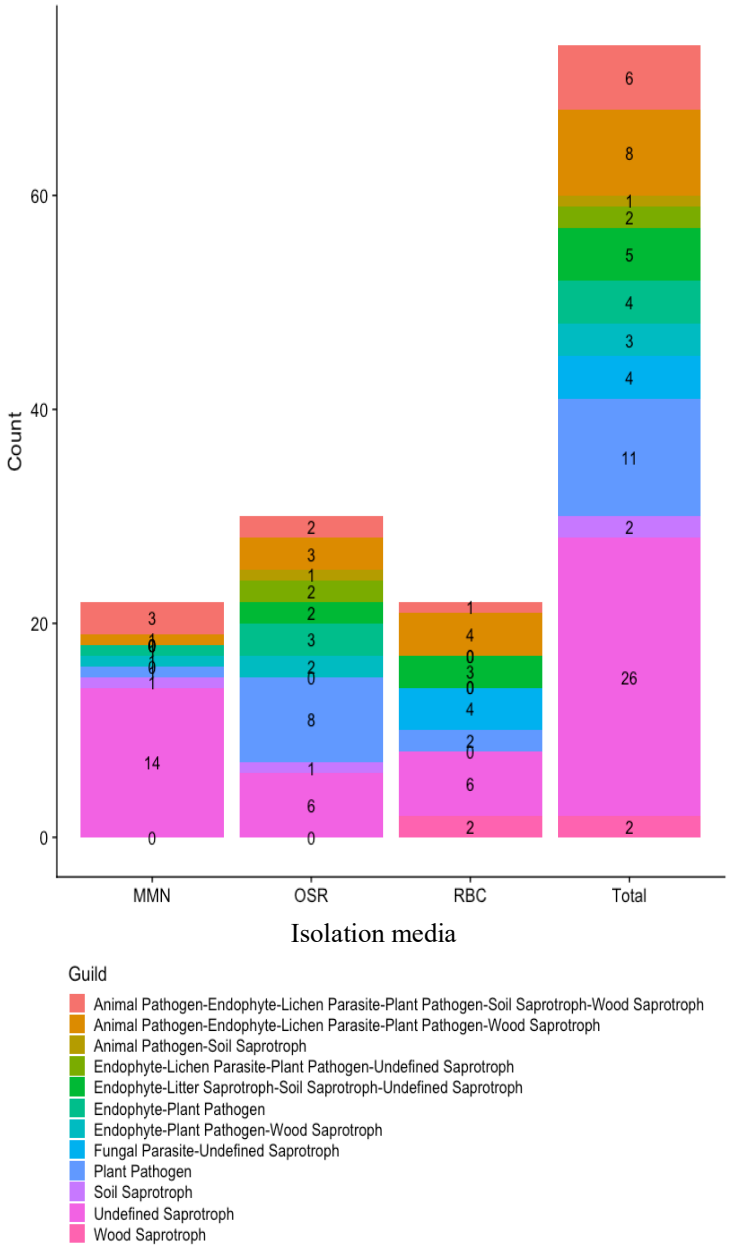

Figure 3.4 Stacked bar plot of FunGuild assigned Guilds for culture collection isolates. *Counts of isolate guilds by culture medium and total collection. MMN = Modified Melin-Norkrans Agar, OSR = Oilseed Rape Extract Agar, RBC = Rose Bengal Chloramphenicol Agar, Total = All isolation media combined. Numbers are representative of count of isolates with each guild assignment. N = 74.*

Table 3.4 FunGuild assignments of culture collection isolates. *Isolate taxonomic ID, guild assignment confidence as determined by FunGuild analysis and assigned guilds.*

isolates	isolate taxonomic id	confidence	guild
RFMMN4	*Fusarium asiaticum*	Possible	Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Soil Saprotroph-Wood Saprotroph
RFMMN14	*Fusarium culmorum*	Possible	
RFMMN5	*Fusarium sp.*	Possible	
RFOSR4	*Fusarium sp.*	Possible	
RFRBC7	*Fusarium sp.*	Possible	
RFOSR17	*Fusarium tricinctum*	Possible	
RHZRBCX1	*Cladosporium anthropophilum*	Possible	Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Wood Saprotroph
RHZRBCX2	*Cladosporium cf. subuliforme*	Possible	
RHZDI3OSRAA2	*Cladosporium sp.*	Possible	
WRFOSRA1	*Cladosporium sp.*	Possible	
RHZDI3RBC3	*Cryptococcus sp. HB 982*	Possible	
RHZOSRX1	*Leptosphaeria biglobosa brassicae*	Possible	
RHZDI4RBC3	*Rhizopus stolonifer*	Possible	
RHZDI1MMNX	*Trichoderma koningiopsis*	Possible	
WRODI2OSR2	*Trichoderma sp.*	Possible	
WRODI2RBC1	*Trichoderma sp.*	Possible	
RHZDI2OSR1	*Neonectria sp.*	Probable	Endophyte-Lichen Parasite-Plant Pathogen-Undefined Saprotroph
RHZDI3OSRAA1	Uncultured psiloglonium	Probable	
RFOSR20	*Mortierella elongata*	Possible	Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph
RFRBC4	Uncultured Mortierella	Possible	

RFRBC6	Uncultured Mortierella	Possible	
RHZDI3OSR3.1	Uncultured Mortierella	Possible	
RHZDI2RBC4	Uncultured Zygomycete	Possible	
RFMMN3.4	*Trichoderma sp. A11*	Probable	Endophyte-Plant Pathogen
RFOSR11	Uncultured Michrodochium	Possible	
RFOSR18	Uncultured Michrodochium	Possible	
RFOSR19	Uncultured Michrodochium	Possible	
SRFOSR2	*Dendryphion nanum*	Probable	Endophyte-Plant Pathogen-Wood Saprotroph
RFMMN2	*Phomopsis sp.*	Probable	
RFOSR7	*Phomopsis sp.*	Probable	
RHZDI2RBC2.1	*Cystofilobasidium macerans*	Possible	Fungal Parasite-Undefined Saprotroph
RHZDI2RBC2.2	*Cystofilobasidium macerans*	Possible	
RHZDI3RBC2	*Leptosphaeria biglobosa brassicae*	Possible	
RHZDI2RBCX1	Uncultured Cryptococcus	Possible	
RHZDI4OSR1	*Cladosporium cladosporoides*	Probable	Plant Pathogen
RFOSRXC	*Leptosphaeria biglobosa brassicae*	Probable	
RHZDI4OSR2	*Leptosphaeria biglobosa brassicae*	Probable	
RFOSR9	*Leptosphaeria biglobosa brassicae*	Probable	
RHZDIRBC4	*Mucor hiemalis*	Probable	
RFOSR3	*Neonectria sp.*	Probable	
RFMMN12	*Penicillium aurantiogriseum*	Probable	
RHZRBCX5	*Scytalidium lignicola*	Probable	
RFOSR5	Uncultured Dendryphion	Probable	

WRODI2MMN1	*Geomyces pannorum*	Probable	Soil Saprotroph
RFOSR1	*Geomyces pannorum*	Probable	
SRFOSR3	*Phomopsis sp.*	Probable	
RHZDI4XAMMN	*Cladosporium cladosporoides*	Probable	Undefined Saprotroph
RFMMN13	*Dactylionectria hordeicola*	Possible	
RFMMN8	*Dactylionectria hordeicola*	Possible	
RFOSRAA1	*Dactylonectria hordeicola*	Possible	
RFRBC1	*Dactylonectria hordeicola*	Possible	
RFMMN11	*Harzia valata*	Probable	
RHZDI2OSR2	*Harzia valata*	Probable	
RHZDI2MMN5	*Juxtiphoma eupyrena*	Probable	
RHZDI3RBC1	*Leptosphaeria biglobosa brassicae*	Possible	
RHZDI4RBC1	*Leptosphaeria biglobosa brassicae*	Possible	
RFMMN7	*Mucor circinelloides*	Probable	
RHZDI2MMN1	*Mucor hiemalis*	Possible	
RHZDI3OSR3.2	*Mucor hiemalis*	Probable	
RFMMN10	*Mucor sp.*	Probable	
RHZDI3OSRXB	*Penicilium canescens*	Possible	
RFMMN1	*Penicillum sp.*	Possible	
RFMMN9	*Penicillum sp.*	Possible	
WRODI1OSR1	*Pseudogymnoascus pannorum*	Possible	
RFOSR2	*Rhizopus stolonifer*	Possible	
RFMMN3.2	*Trichoderma sp.*	Possible	
RFMMN3.3	*Trichoderma sp.*	Possible	

RFMMN3.1	*Trichoderma sp. isolate yi1147*	Possible	
RHZDI1RBC1	*Trichoderma tomentosum*	Possible	
RFMMNXA	*Trichoderma viride*	Possible	
RFOSR8	*Tetracladium maxilliforme*	Probable	
RHZDI2XDRBC	Uncultured Trichoderma	Possible	
RHZRBCX4	*Cladosporium cf. subuliforme*	Probable	Wood Saprotroph
RFRBC3	*Trametes sp.*	Highly Probable	

3.4.3 Identification of ecologically and functionally relevant culture collection isolates

To determine whether the fungal culture collection presented in this study was representative of UK OSR rhizosphere mycobiomes, isolates were aligned against the fungal OTU database presented in Hilton et al., 2021 study of the UK wide OSR rhizosphere microbiome. Collection isolates were aligned to the ITS OTU fasta file using a NCBI BLAST local alignment for highly similar sequences (Altschul et al., 1990).The data presented in Hilton et al., 2021 covers the bulk soil, rhizosphere soil and root microbiomes of OSR grown on 37 farms across the UK (**Figure 3.5**). This publication also identified rhizosphere fungal taxa which were significantly correlated with OSR yield at a landscape level. This also enabled prioritisation of isolates for co-incubation studies with OSR based on these ecologically relevant (community relative

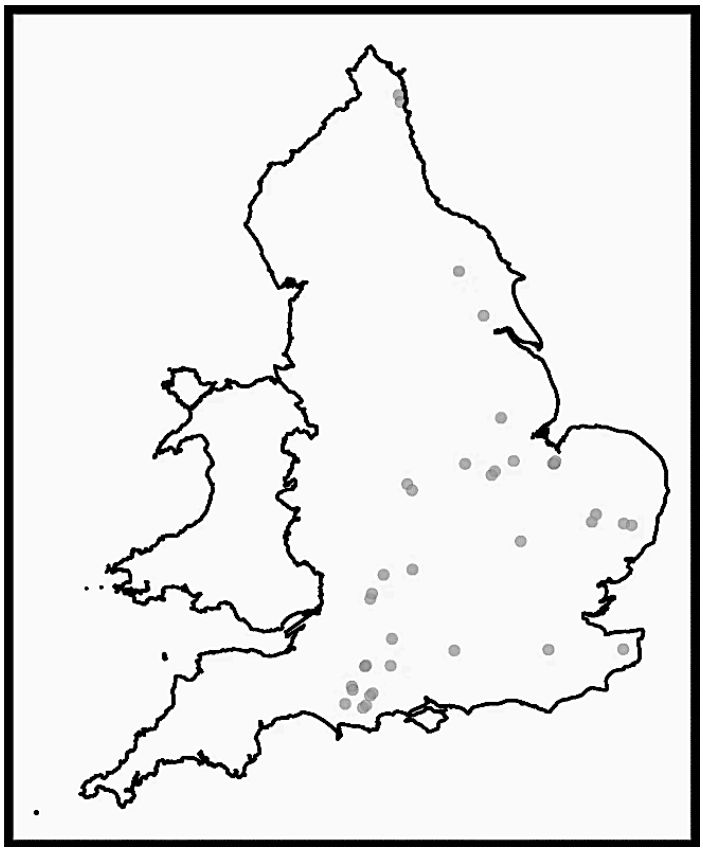

Figure 3.5 Location of sample sites used in Hilton et al. (2021). *Points on map are indicative of the 37 farm sample sites used in Hilton et al. (2021) investigation of UK wide OSR rhizosphere microbiomes. Figure taken from Hilton et al. (2021).*

abundance and landscape distribution) and functionally relevant (significant correlation with OSR biomass) parameters.

All 74 isolates in the culture collection were matched to an OTU present in the UK wide dataset at a similarity level of > 98 %. In total 33 OTUs were matched to culture collection isolates (**Table 3.5**). Of these OTUs, 31 were found in all compartments, whereas OTU 3258 was found in root and rhizosphere soil only, OTU

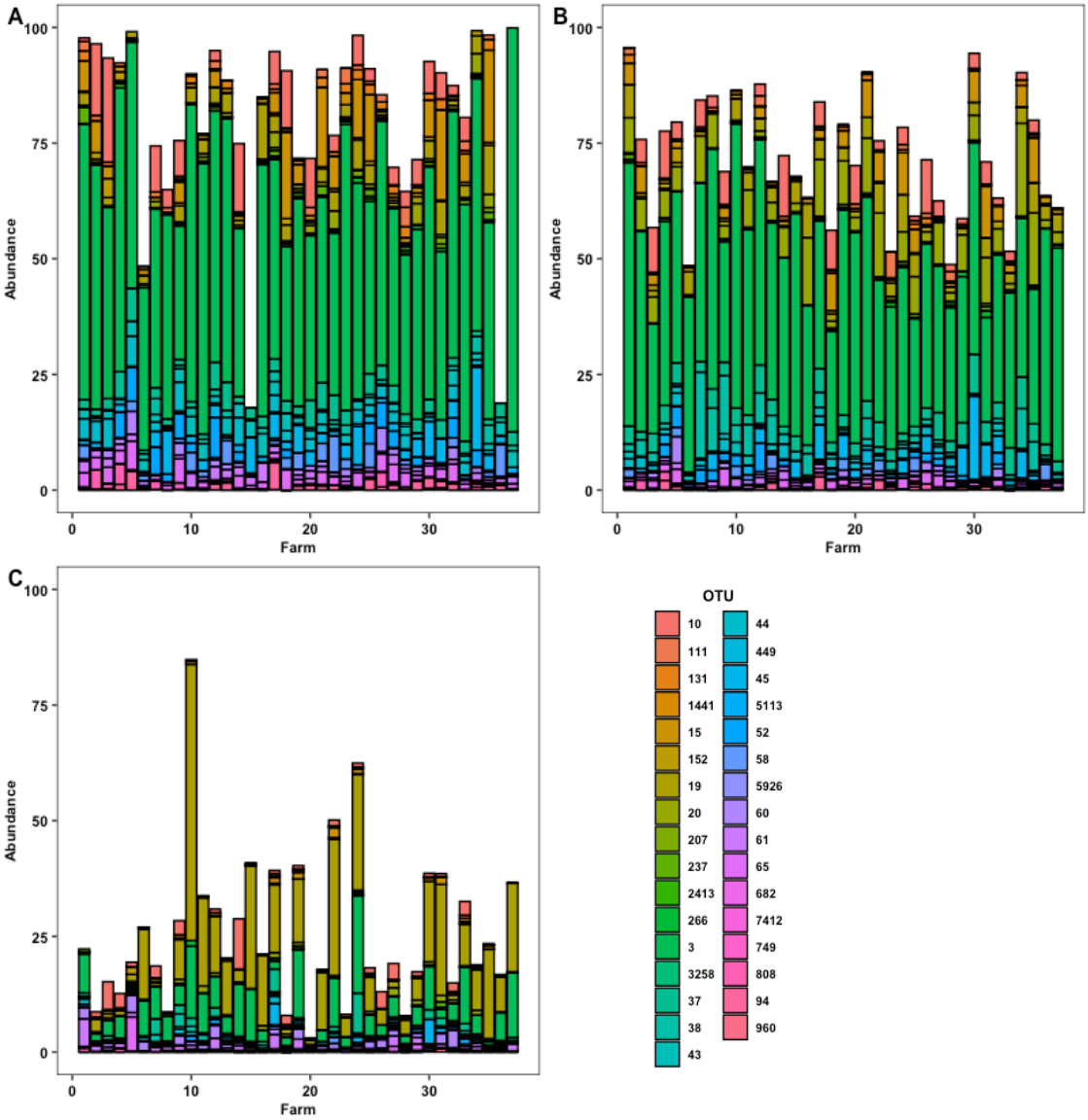

Figure 3.6 Stacked bar plots of isolate collection matched OTUs across UK. *Data are representative of UK wide ITS OTUs presented in (Hilton et al., 2021) filtered by OTUs BLAST matched to the isolate collection presented in this study. Data are mean relative abundance of each isolate matched OTU at each farm site sampled in Hilton et al., 2021.* ***A****) Bulk Soil,* ***B****) Rhizosphere Soil,* ***C****) Root. N = 37.*

5113 in bulk soil and rhizosphere soil only and OTU 7412 only found in rhizosphere soil (**Tables 3.6, 3.7 & 3.8**). All OTUs shifted in relative abundance and detection frequency between root, rhizosphere soil and bulk soil compartments (**Figures 3.6 & 3.7**) (**Tables 3.6, 3.7 & 3.8**). When combined, culture collection matched isolates represented 88.2 % of bulk soil, 79.1 % of rhizosphere soil and 28.0 % of root mycobiome relative abundance. This decrease in relative abundance moving from bulk soil to rhizosphere soil to root mycobiome indicated that the culture collection was able to capture taxa which are more abundant in bulk soil and rhizosphere soil ecological niches compared to the root ecological niche.

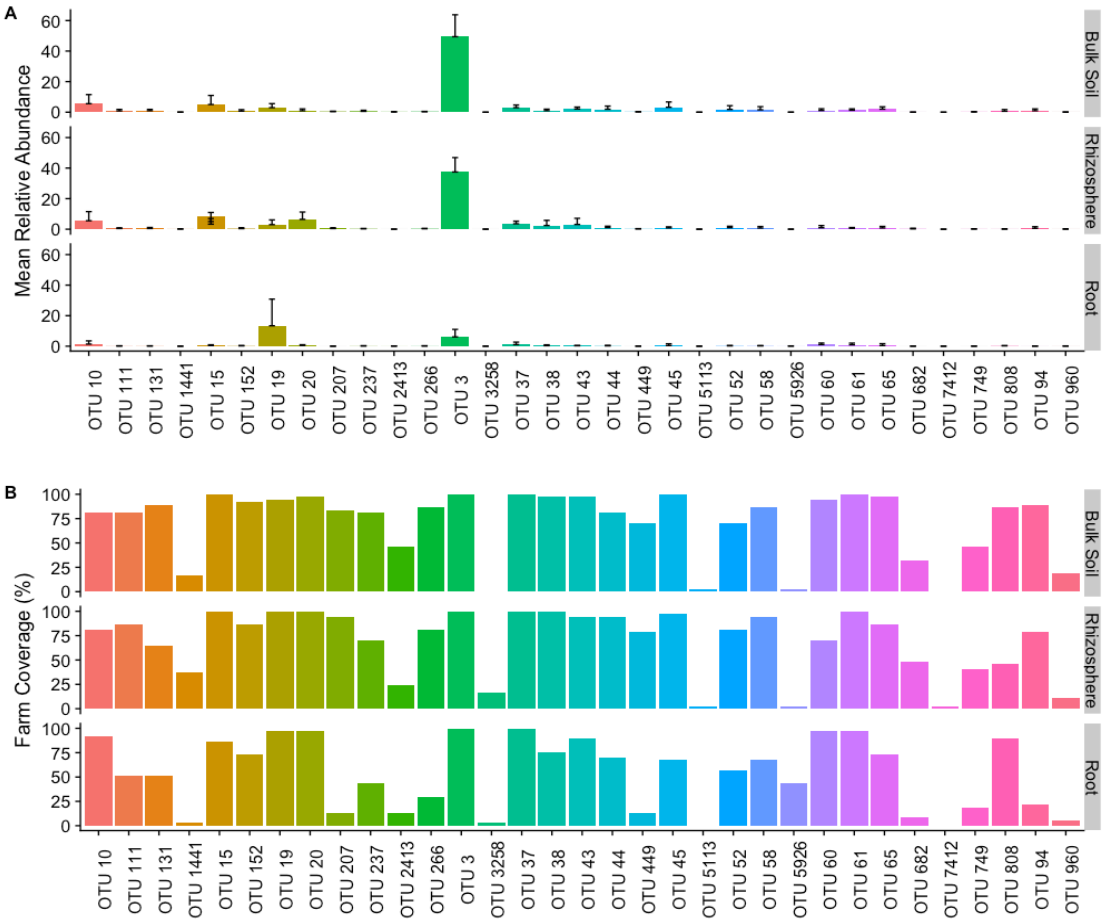

Figure 3.7 Culture collection matched OTUs average relative abundancies and detection in UK wide OSR bulk soil, rhizosphere soil and roots. *Bar plots are representative of **A)** average relative abundance across farms and **B)** percentage of farms matched OTUs present in. Data are representative of UK wide ITS OTUs presented in Hilton et al., 2021 filtered by OTUs BLAST matched to the isolate collection presented in this study. Data are mean relative abundance of each isolate matched OTU at each farm site sampled in Hilton et al. (2021). OTU at each farm site sampled in Hilton et al. (2021).*

Table 3.5 Culture collection isolates matched to UK wide OSR ITS OTUs. *Isolate ITS sequences were aligned with OTUs present in the ITS dataset presented in Hilton et al. (2021) using NCBI BLAST with the ITS dataset presented in Hilton et al. (2021) as a local database. Table denotes fungal collection isolates matched to Hilton et al. (2021) ITS OTUs with a BLAST identity score > 99 %.*

otu	otu taxonomic id	isolates	isolate taxonomic id
3	(g) Mortierella	RFRBC4	*Uncultured Mortierella*
		RHZDI3OSR3.1	*Uncultured Mortierella*
10	(g) Mortierella	RFOSR20	*Mortierella elongata*
15	(f) Pleosporaceae	RHZDI2MMN5	*Juxtiphoma eupyrena*
		RHZRBCX5	*Scytalidium lignicola*
		RHZDI3OSRAA1	*Uncultured psiloglonium*
19	(s) *Tetracladium maxilliforme*	RFOSR8	*Tetracladium maxilliforme*
20	(c) Tremellomycetes	RHZDI2RBCX1	*Uncultured Cryptococcus*
		RHZDI3RBC3	*Cryptococcus sp. HB 982*
37	(f) Davidiellaceae	RHZRBCX1	*Cladosporium anthropophilum*
		RHZRBCX2	*Cladosporium cf. subuliforme*
		RHZRBCX4	*Cladosporium cf. subuliforme*
		RHZDI4XAMMN	*Cladosporium cladosporoides*
		RHZDI4OSR1	*Cladosporium cladosporoides*
		RHZDI3OSRAA2	*Cladosporium sp.*
		WRFOSRA1	*Cladosporium sp.*
38	(c) Tremellomycetes	RHZDI2RBC2.1	*Cystofilobasidium macerans*
		RHZDI2RBC2.2	*Cystofilobasidium macerans*
43	(g) Microdochium	RFOSR19	*Uncultured Michrodochium*
		RFOSR18	*Uncultured Michrodochium*
		RFOSR11	*Uncultured Michrodochium*
44	(p) Ascomycota	RHZDI4RBC3	*Rhizopus stolonifer*
		RFOSR2	*Rhizopus stolonifer*
45	(f) Nectriaceae	RFMMN4	*Fusarium asiaticum*
		RFOSR4	*Fusarium sp.*
		RFMMN14	*Fusarium culmorum*
52	(o) Hypocreales	RFMMN5	*Fusarium sp.*
58	(f) Nectriaceae	RFRBC7	*Fusarium sp.*
60	(f) Pleosporaceae	SRFOSR2	*Dendryphion nanum*
		RFOSR5	*Uncultured Dendryphion*
61	(f) Hypocreales	RFMMN8	*Dactylionectria hordeicola*
		RFMMN13	*Dactylionectria hordeicola*
		RFRBC1	*Dactylonectria hordeicola*
		RFOSRAA1	*Dactylonectria hordeicola*

65	(f) Mortierellaceae	RHZDI2RBC4	*Uncultured Zygomycete*
94	(o) Leotiomycetes	RFOSR1	*Geomyces pannorum*
		WRODI1OSR1	*Pseudogymnoascus pannorum*
		WRODI2MMN1	*Geomyces pannorum*
111	(f) Hypocreaceae	RFMMN3.3	*Trichoderma sp.*
		RFMMN3.1	*Trichoderma sp. isolate yi1147*
		RFMMN3.2	*Trichoderma sp.*
		RFMMN3.4	*Trichoderma sp. A11*
		RHZDI1MMNX	*Trichoderma koningiopsis*
		WRODI2OSR2	*Trichoderma sp.*
		RFMMNXA	*Trichoderma viride*
		WRODI2RBC1	*Trichoderma sp.*
131	(f) Hypocreaceae	RHZDI2XDRBC	*Uncultured Trichoderma*
152	(g) Ilyonectria	RFMMN11	*Harzia valata*
		RHZDI2OSR2	*Harzia valata*
207	(f) Trichocomaceae	RHZDI3OSRXB	*Penicilium canescens*
237	(f) Nectriaceae	RFOSR17	*Fusarium tricinctum*
266	(f) Mucoraceae	RHZDI2MMN1	*Mucor hiemalis*
		RHZDI3OSR3.2	*Mucor hiemalis*
		RHZDIRBC4	*Mucor hiemalis*
449	(f) Trichocomaceae	RFMMN12	*Penicillium aurantiogriseum*
682	(o) Eurotiales	RHZOSRX1	*Leptosphaeria biglobosa brassicae*
		RHZDI3RBC2	*Leptosphaeria biglobosa brassicae*
		RHZDI4RBC1	*Leptosphaeria biglobosa brassicae*
		RFOSR9	*Leptosphaeria biglobosa brassicae*
		RHZDI4OSR2	*Leptosphaeria biglobosa brassicae*
		RFOSRXC	*Leptosphaeria biglobosa brassicae*
		RHZDI3RBC1	*Leptosphaeria biglobosa brassicae*
749	(g) Penicillium	RFMMN9	*Penicillum sp.*
808	(g) Mortierella	RFRBC6	*Uncultured Mortierella*
960	(g) Mucor	RFMMN7	*Mucor circinelloides*
1441	(g) Phomopsis	SRFOSR3	*Phomopsis sp.*
		RFMMN2	*Phomopsis sp.*
		RFOSR7	*Phomopsis sp.*
2413	(f) Hypocreaceae	RHZDI1RBC1	*Trichoderma tomentosum*
3258	(s) Mucor hiemalis	RFMMN10	*Mucor sp.*
5113	(g) Penicillium	RFMMN1	*Penicillum sp.*
	(g) Ilyonectria	RHZDI2OSR1	*Neonectria sp.*

5926		RFOSR3	*Neonectria sp.*
7412	(s) *Trametes versicolor*	RFRBC3	*Trametes sp.*

Table 3.6 Culture Collection matched OTU statistics for root compartment. Isolates ordered top to bottom by average relative abundance.

otu	average relative abundance (%)	stdev (%)	farm coverage (%)	otu taxonomic id
19	13.373	17.446	97.297	(s) *Tetracladium maxilliforme*
3	6.119	4.911	100.000	(g) Mortierella
10	1.382	2.100	91.892	(g) Mortierella
60	1.088	0.992	97.297	(f) Pleosporaceae
37	1.050	1.633	100.000	(f) Davidiellaceae
61	0.916	1.119	97.297	(f) Hypocreales (fam Incertae sedis)
20	0.543	0.431	97.297	(c) Tremellomycetes
45	0.441	1.125	67.568	(f) Nectriaceae
15	0.439	0.498	86.486	(f) Pleosporaceae
65	0.434	1.219	72.973	(f) Mortierellaceae
38	0.347	0.549	75.676	(c) Tremellomycetes
43	0.286	0.247	89.189	(g) Microdochium
44	0.224	0.361	70.270	(p) Ascomycota
808	0.208	0.211	89.189	(g) Mortierella
52	0.207	0.324	56.757	(o) Hypocreales
152	0.205	0.281	72.973	(g) Ilyonectria
58	0.178	0.268	67.568	(f) Nectriaceae
237	0.103	0.230	43.243	(f) Nectriaceae
131	0.101	0.160	51.351	(f) Hypocreaceae
111	0.097	0.172	51.351	(f) Hypocreaceae
266	0.081	0.218	29.730	(f) Mucoraceae
5926	0.051	0.113	43.243	(g) Ilyonectria
2413	0.034	0.115	13.514	(f) Hypocreaceae
960	0.030	0.139	5.405	(g) Mucor
94	0.028	0.067	21.622	(o) Leotiomycetes (ord Incertae sedis)
749	0.019	0.043	18.919	(g) Penicillium
449	0.012	0.034	13.514	(f) Trichocomaceae
682	0.011	0.041	8.108	(o) Eurotiales
207	0.009	0.028	13.514	(f) Trichocomaceae
1441	0.001	0.008	2.703	(g) Phomopsis
3258	0.001	0.008	2.703	(s) *Mucor hiemalis*
5113	0.000	0.000	0.000	(g) Penicillium
7412	0.000	0.000	0.000	(s) *Trametes versicolor*

Table 3.7 Culture Collection matched OTU statistics for rhizosphere compartment. Isolates ordered top to bottom by largest average relative abundance.

otu	mean relative abundance (%)	stdev (%)	farm coverage (%)	otu taxonomic id
3	37.466	9.422	100.000	(g) Mortierella
20	6.438	4.778	100.000	(c) Tremellomycetes
10	5.550	5.941	81.081	(g) Mortierella
15	5.009	5.930	100.000	(f) Pleosporaceae
37	3.550	1.695	100.000	(f) Davidiellaceae
15	3.293	3.674	72.973	(f) Pleosporaceae
43	3.089	4.021	94.595	(g) Microdochium
19	2.974	3.105	100.000	(s) *Tetracladium maxilliforme*
38	2.407	3.370	100.000	(c) Tremellomycetes
44	1.041	0.932	94.595	(p) Ascomycota
52	0.991	0.946	81.081	(o) Hypocreales
60	0.939	1.418	70.270	(f) Pleosporaceae
45	0.832	0.702	97.297	(f) Nectriaceae
65	0.804	1.016	86.486	(f) Mortierellaceae
58	0.757	0.969	94.595	(f) Nectriaceae
61	0.611	0.478	100.000	(f) Hypocreales (fam Incertae sedis)
94	0.605	1.004	78.378	(o) Leotiomycetes (ord Incertae sedis)
111	0.447	0.431	86.486	(f) Hypocreaceae
131	0.443	0.682	64.865	(f) Hypocreaceae
207	0.427	0.470	94.595	(f) Trichocomaceae
152	0.355	0.568	86.486	(g) Ilyonectria
266	0.249	0.313	81.081	(f) Mucoraceae
237	0.222	0.272	70.270	(f) Nectriaceae
449	0.161	0.189	78.378	(f) Trichocomaceae
682	0.159	0.501	48.649	(o) Eurotiales
749	0.076	0.136	40.541	(g) Penicillium
808	0.062	0.109	45.946	(g) Mortierella
1441	0.061	0.126	37.838	(g) Phomopsis
960	0.034	0.139	10.811	(g) Mucor
2413	0.016	0.033	24.324	(f) Hypocreaceae
3258	0.014	0.035	16.216	(s) *Mucor hiemalis*
5113	0.003	0.016	2.703	(g) Penicillium
5926	0.001	0.008	2.703	(g) Ilyonectria
7412	0.001	0.008	2.703	(s) *Trametes versicolor*

Table 3.8 Culture Collection matched OTU statistics for bulk soil compartment. Isolates ordered top to bottom by largest average relative abundance.

otu	mean relative abundance (%)	stdev (%)	farm coverage (%)	otu taxonomic id
3	49.419	14.361	100.000	(g) Mortierella
10	5.550	5.941	81.081	(g) Mortierella
15	5.009	5.930	100.000	(f) Pleosporaceae
45	3.189	3.464	100.000	(f) Nectriaceae
37	2.950	1.743	100.000	(f) Davidiellaceae
19	2.950	2.636	94.595	(s) *Tetracladium maxilliforme*
43	2.092	1.233	97.297	(g) Microdochium
65	1.957	1.558	97.297	(f) Mortierellaceae
44	1.919	2.052	81.081	(p) Ascomycota
52	1.785	2.467	70.270	(o) Hypocreales
58	1.547	2.049	86.486	(f) Nectriaceae
61	1.281	0.880	100.000	(f) Hypocreales (fam Incertae sedis)
60	1.095	1.143	94.595	(f) Pleosporaceae
38	0.970	1.005	97.297	(c) Tremellomycetes
131	0.947	0.846	89.189	(f) Hypocreaceae
94	0.938	1.201	89.189	(o) Leotiomycetes (ord Incertae sedis)
20	0.926	1.219	97.297	(c) Tremellomycetes
111	0.818	0.974	81.081	(f) Hypocreaceae
152	0.728	0.909	91.892	(g) Ilyonectria
808	0.614	1.076	86.486	(g) Mortierella
237	0.527	0.705	81.081	(f) Nectriaceae
207	0.308	0.342	83.784	(f) Trichocomaceae
266	0.246	0.246	86.486	(f) Mucoraceae
449	0.146	0.191	70.270	(f) Trichocomaceae
2413	0.115	0.217	45.946	(f) Hypocreaceae
749	0.108	0.245	45.946	(g) Penicillium
960	0.042	0.115	18.919	(g) Mucor
682	0.028	0.051	32.432	(o) Eurotiales
1441	0.026	0.074	16.216	(g) Phomopsis
5113	0.001	0.008	2.703	(g) Penicillium
5926	0.001	0.008	2.703	(g) Ilyonectria
3258	0.000	0.000	0.000	(s) *Mucor hiemalis*
7412	0.000	0.000	0.000	(s) *Trametes versicolor*

However, the culture collection was able to capture isolates which were highly abundant and highly dispersed across the UK OSR rhizosphere mycobiome. The OTU with the highest abundance in the bulk soil and rhizosphere samples was OTU 3 with an average relative abundance of 49.5 % and 37.5 % (respectively). OTU 3 was also present in a 100 % of samples in both compartments (**Figure 3.6 & 3.7**) (**Tables 3.7 & 3.8**). OTU 3 had a taxonomic assignment at the genus level of Mortierella and was matched to two isolates in the culture collection (**Table 3.5**), namely, isolates RFRBC4 and RHZDI3OSR3.1 which were both assigned as 'Unidentified Mortierella'. In the root compartment, OTU 19 had the highest average relative abundance of 13.4 % and was detected in 97.3 % of samples. This OTU had a taxonomic assignment at the species level of *Tetracladium maxilliforme* and was matched to one isolate in the culture collection, namely, RFOSR8 which was assigned as 'Unidentified Tetracladium'. OTU 19 was also identified as being significantly positively correlated with yield (**Table 3.9**). In total, five OTUs which were matched to ten culture collection isolates were identified as being significantly correlated with yield in Hilton et al., 2021 (**Table 3.9)**.

Table 3.9 OTUs correlated with yield from (Hilton et al., 2021).

compartment	otu	yield correlation (p < 0.05)	matched isolates	taxonomic id
root	19	0.281	RFOSR8	Uncultured Tetracladium
rhizosphere soil	45	0.22	RFMMN4	*Fusarium asiaticum*
			RFOSR4	*Fusarium sp.*
			RFMMN14	*Fusarium culmorum*
	58	-0.178	RFRBC7	*Fusarium sp.*
bulk soil	43	-0.252	RFOSR19	Uncultured Michrodochium
			RFOSR18	Uncultured Michrodochium
			RFOSR11	Uncultured Michrodochium
	58	-0.18	RFRBC7	*Fusarium sp.*
	38	-0.266	RHZDI2RBC2.1	*Cystofilobasidium macerans*
			RHZDI2RBC2.2	*Cystofilobasidium macerans*

3.4.4 Assessment of isolate function using OSR – isolate co-incubation

3.4.4.1 Selection of isolates for co-incubation studies

In total, 13 isolates were identified as priorities for co-incubation studies based on taxa abundance, distribution, and relationship to OSR yield. These isolates included those matched to OTU3, OTU19, OTU45, OTU58, OTU43, OTU58 and OTU38. Isolates matched to OTU3 (RFRBC4 & RHZDI3OSR3.1), were chosen due to OTU3 being the most abundant and distributed taxa of OSR bulk soil and rhizosphere soil mycobiomes (**Figure 3.7**). The OTU19 matched isolate RFOSR8 was chosen due to OTU19 being the most abundant and distributed taxa in OSR root isolate mycobiomes (**Figure 3.7**), and due to its positive correlation with OSR yields (**Table 3.9**). Isolates matched to OTUs 45, 58, 43, 58 and 38 (RFMMN4, RFOSR4, RFMMN14, RFRBC7, RFOSR19, RFOSR18, RFOSR11, RFRBC7, RHZDI2RBC2.1 and RHZDI2RBC2.2) were all chosen for priority due to their significant correlation to OSR yields (**Table 3.9**). Of these 13 isolates, 4 were successfully screened in OSR co-incubation studies including isolates, RFOSR11, RFOSR18, RFOSR8, RFRBC4 and RHZDI3OSR3.1.

In addition to these isolates, 13 isolates were also chosen based on FunGuild assignment and known interactions with OSR from literature. Isolates SRFOSR2, SRFOSR3, RFMMN2, RFOSR7 and WRODI2OSR2 were chosen due to their FunGuild guild assignments having either endophyte and/or plant pathogen in their functional assignment. RFMMN12 and RFMMN9 were chosen due to their assignments as plant pathogens. RFOSR1, RFOSR2, RFOSR15 and RFMMN3.3 were chosen due to their assignment as undefined saprotrophs. Finally, RHZDI3RBC1 and RHZDI3RBC2 were chosen due to their taxonomic assignment as *L. biglobosa brassicae*, a taxon which is known as a major pathogen of OSR and has been observed to significantly increase plant biomass in OSR.

3.4.4.2 Co-incubation studies

Prioritised culture collection isolates were co-inoculated with OSR seeds to determine if fungal isolates had significant impacts upon OSR seedling biomass in terms of total plant dry weight and root shoot ratio. These co-inoculated studies run for a 4-week period and as such only observed isolate impact on OSR during early life

stages. This screening method provided an insight as to whether fungal isolates may have a beneficial or deleterious function in OSR microbiomes in terms of OSR biomass and health.

Of the 19 fungal isolates screened in this study, only 6 were identified to have a significant impact on OSR biomass and 1 identified as having a significant impact on OSR root shoot ratio (**Figure 3.8 & 3.9**). Isolates impacting biomass were RHZDI3OSR3.1, RHZDI3RBC1, RHZDI3RBC2, RFMMN9, RFOSR7 and RFOSR1. RHZDI3RBC1 was also identified as having a significant impact on root shoot ratio. All of these isolates had a deleterious impact upon OSR with significantly lower biomass and root shoot ratio compared to both controls. Only one prioritised isolate was identified as having a significant impact on OSR biomass. Isolate RHZDI3OSR3.1 (Uncultured Mortierella) was prioritised for its alignment with OTU3, the isolate with the highest relative abundance in UK OSR bulk soil and rhizosphere soil.

No significant differences between controls were observed, however, significant differences between isolate treated and either untreated control or sterile barley control were observed (**Figure 3.8 & 3.9**). Isolates, WRODI2OSR2, SRFOSR3 and RFOSR15 had significant differences in root shoot ratio compared to the sterile barley control. RFMMN12 had a significant difference in biomass compared to the barley control and RFMMN3.3 the uninoculated control. Differences in biomass achieved between batches was also observed with isolates screened in batch 2 showing lower biomass weights than batch 1(**Figure 3.8 & 3.9**). This indicated that batch to batch differences and variation in control biomass occurred in addition to observed isolate effects.

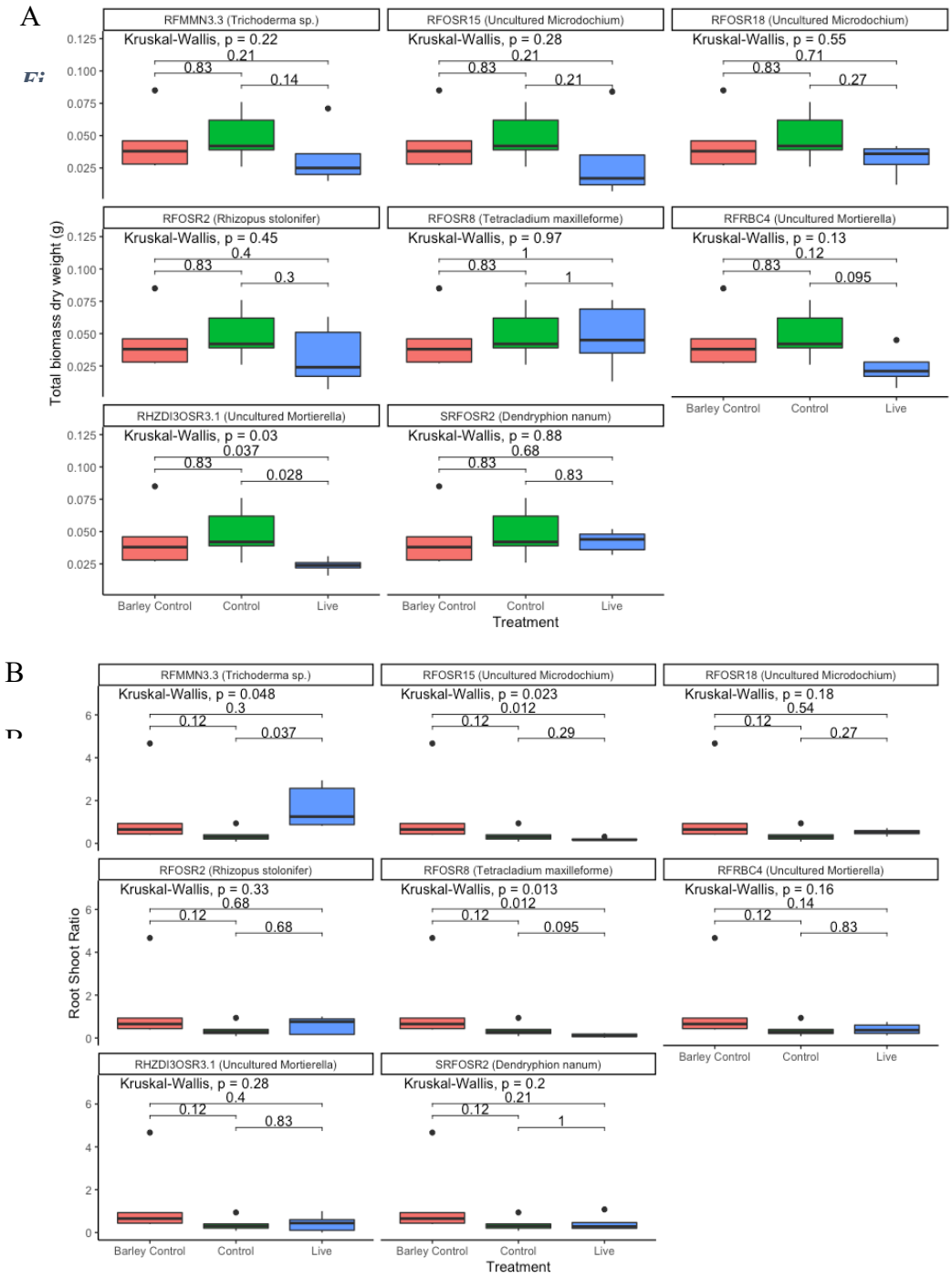

***Figure 3.8 Co-incubation study OSR biomass data (batch 1)**. Data are representative total plant dry weight **A)** and **B)** root shoot ratio of OSR treated with uninoculated barley (Barley Control), no inoculum (Control) and barley inoculated with a culture collection isolate (live). Graphs are faceted by culture collection isolate with statistical analysis being carried out for each individual isolate. Statistical analysis comprised of Kruskal-Wallis analysis (noted on each graph) with Dunns' post hoc analysis with Bonferroni multiple comparison correction (noted on graphs above comparison lines). Outliers identified by IQR are denoted as datapoints on graphs. N = 5.*

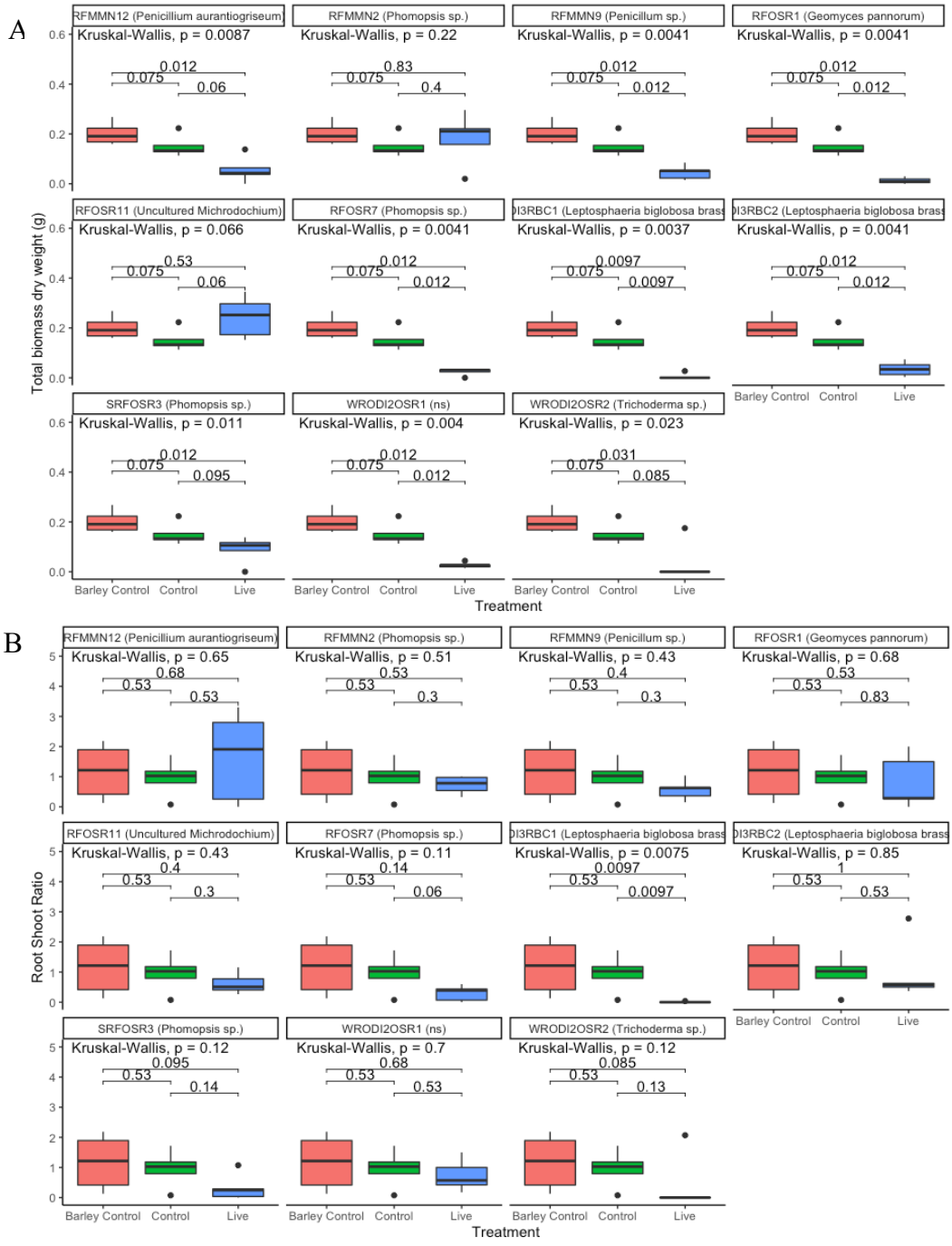

Figure 3.9 Co-incubation study OSR biomass data (batch 2). *Data are representative total plant dry weight* ***A)*** *and* ***B)*** *root shoot ratio of OSR treated with uninoculated barley (Barley Control), no inoculum (Control) and barley inoculated with a culture collection isolate (live). Graphs are faceted by culture collection isolate with statistical analysis being carried out for each individual isolate. Statistical analysis comprised of Kruskal-Wallis analysis (noted on each graph) with Dunns' post hoc analysis with Bonferroni multiple comparison correction (noted on graphs above comparison lines). Outliers identified by IQR are denoted as datapoints on graphs. N = 5.).*

3.5 Discussion

In this study we present the most comprehensive functional analysis of OSR rhizosphere mycobiomes to date through the isolation and investigation of a diverse fungal culture collection. This culture collection was isolated from UK OSR rhizosphere soil and root tissues on a nutritionally diverse selection of isolation media. In total, the collection comprised of 74 isolates with a diverse range of taxonomic and fungal functional guilds being represented. Function guild assignments included individual and mixed assignments of, saprotrophs, pathogens, and endophytes. However, most functional guild assignments were given the lowest possible confidence rating, indicating the current limitations of this functional assignment technique. However, a number of taxa which are known to be functionally important pathogens of OSR or potentially beneficial endophytes were isolated including, Leptosphaeria, Fusarium and Trichoderma species (AHDB, 2021, Card et al., 2015). This culture collection was also comprised of taxa commonly found across the UK's OSR rhizosphere mycobiome and included several taxa significantly correlated with OSR yield at a UK landscape level (Hilton et al., 2021). This information in addition to taxonomic ID and FunGuild functional guild assignment was then used to identify isolates for priority in co-incubation studies with OSR.

Co-incubation studies identified 6 isolates which had a significant deleterious impact on OSR seedling biomass after 4 weeks. These isolates included a highly abundant and distributed taxon in OSR rhizosphere microbiomes (Uncultured Mortierella), known OSR and plant pathogens (*L. biglobosa brassicae*, *Phomopsis sp.* & *Penicillum sp.*) and an 'undefined saprotroph' (*G. pannorum*). Interestingly, another OTU related to the Uncultured Mortierella isolate and isolates significantly correlated with OSR yields (*T. maxilleforme* & Uncultured Microdochium) had no significant impact on biomass. This may indicate species specific effects on biomass and alternative mechanisms for impacting yield other than impacts on OSR biomass during the early growth stages. Together, these analyses helped to identify several taxa of interest for further investigation including sequencing of whole isolate genomes and plant-microbe interaction studies to unravel the specific interactions these fungi have with OSR.

3.5.1 Inference of functional assignment based on taxonomy

Culture collection isolates were assigned functional guilds using FunGuild functional analysis. This analysis method aims to assign ecological guilds and trophic modes to fungal taxa based on their taxonomic assignments and provides a confidence level of this assignment. Isolates were assigned to a range of guilds including individual function guilds such as 'Plant Pathogen', 'Soil Saprotroph', 'Undefined Saprotroph' and 'Wood Saprotroph', and multiple function guilds including mixtures of symbiotic (endophyte), pathogenic (animal pathogen, plant pathogen, parasite) and saprotrophic (wood saprotroph, soil saprotroph, litter saprotroph) guilds. Most isolates identified in this study were assigned functional guilds at the lowest level of confidence provided by FunGuild, with 46 'possible' assignments. This was followed by 27 isolates with 'probable' assignments and 1 'highly probably' assignment.

The combination of multi-function guild assignment and low confidence of assignments found in this study is indicative of the major constraints of assigning ecological functionality of fungi using taxonomy alone. Indeed, the seminal paper for the FunGuild database identifies three key limitations to FunGuild analysis (Nguyen et al., 2016b). Firstly, population of the database to date has been limited to descriptions of twelve functional guilds. Subdivision of guilds such as 'undefined saprotroph' is required to sufficiently capture the functional diversity of fungi beyond well documented guilds. Secondly, most taxa included in the FunGuild database are assigned to the genus level, reducing the probability of correct guild assignment as species level assignments are most accurate. Approximately 148,000 fungal species have been taxonomically described. Estimates of the total number of species are between 2.2 to 3.8 million and up to 12 million in the most recent estimates (Hawksworth and Lücking, 2017, Wu et al., 2019). Therefore, this low level of taxonomic coverage limits the ability of FunGuild assignment techniques. Thirdly, the lack of functional ecological description of fungal taxa in general limits the use of functional assignment based on sequence data alone. Therefore, studies such as this, which integrate community sequencing data, isolate cultivation and experimentation are necessary to, develop our understanding of fungal ecology in the genomics era (Nguyen et al., 2016b, Peay, 2014).

3.5.2 Creation of a diverse and representative OSR fungal isolate collection

The culture collection presented in this study comprised of isolates representative of taxa commonly found across the UKs OSR rhizosphere mycobiome. This finding was achieved through referencing isolate sequences against fungal OTU sequences from Hilton et al. (2021)'s investigation of OSR rhizosphere microbiome assembly across the UK. All isolates in the culture collection were matched to an OTU from the UK OSR mycobiome dataset with 33 matched OTUs identified. These OTUs were found to represent the majority of UK OSR bulk soil and rhizosphere soil mycobiomes in terms of abundance and distribution. These OTUs did not represent the majority of root mycobiomes in terms of abundance, however, most root taxa were captured. This was due to many root microbiome samples having a high relative abundance of a single species not isolated in this study: *O. brassicae* (Hilton et al., 2021). However, an isolate matched to the second most abundant root OTU identified by Hilton et al. (2021) was isolated (*T. maxilliforme*). In addition, taxa which are commonly associated with OSR across the globe including members of the: Mortierella, Leptosphaeria, Fusarium, Trichoderma and Phomopsis genera were captured in the culture collection. Members of these genera have previously been identified as beneficial endophytes (Card et al., 2015) and pathogens of OSR (AHDB, 2021), indicating that the culture collection was able to capture fungal genera with functional importance in OSR. In addition, 11 culture collection isolates were matched to OSR yield linked OTUs, identifying these isolates as potentially functionally important in OSR and subsequently as a priority for co-incubation studies.

However, whether this isolate collection is fully representative of the UK OSR rhizosphere mycobiome in terms of taxa functionality is debatable given that isolates were obtained from a single UK agricultural field. As previously discussed, different strains of the same microbial species may have significant differences in functional capabilities due to the presence or absence of certain functional genes within accessory genomes (McCarthy and Fitzpatrick, 2019, Sachs et al., 2011). For example, wide variation in intraspecies functionality of mycorrhizal fungi has been observed from strains isolated from different plant hosts, geographic locations and biomes (i.e. arid vs temperate) (Martínez-Garcia et al., 2015, Munkvold et al., 2004). Such strain level variation is not possible to discern from the amplicon sequencing methods used by Hilton et al. (2021) and this study. This precludes the ability to identify if the isolates

presented in this study match the specific strains sequenced in the UK wide survey of OSR rhizosphere microbiomes. Therefore, further isolation attempts from a diverse range of UK OSR fields such as those assessed in Hilton et al. (2021) could provide a more representative collection of UK OSR associated fungi and allow the exploration of strain level differences in functional capabilities.

3.5.3 Co-incubation studies

From a group of 18 successfully screened isolates, 6 were found to have a significant impact on OSR seedling biomass. Inoculation with isolates RFOSR1 (*Geomyces pannorum*), RFOSR7 (*Phomopsis sp*.), RHZDI3RBC1 (*Leptosphaeria biglobosa brassicae*), RHZDI3RBC2 (*L. biglobosa brassicae*), RHZDI3OSR3.1 (Uncultured Mortierella) and RFMMN9 (*Penicillum sp.*) were all associated with significant reductions in dry plant weights compared to controls. A significant reduction in root shoot ratio compared to controls was also observed for RHZDI3RBC1 (*L. biglobosa brassicae*). By comparison isolates matched to OTUs significantly correlated with OSR yields in Hilton et al. (2021) (*T. maxilliforme* & Uncultured Microdochium) had no significant impact on biomass. This may indicate species specific effects on biomass and/or alternative mechanisms for impacting yield other than affecting OSR seedling biomass. As previously discussed in section 3.5.2, strain level differences between fungi can also affect functional capabilities and therefore may also factor into differences in functional capabilities observed between studies.

The biomass impacting isolates identified in this study come from a diverse taxonomic background. Varying levels of research having been carried out on each taxa regarding their functional importance to plants. Isolates RFOSR7 (*Phomopsis sp*.), RHZDI3RBC1 (*L. biglobosa brassicae*), RHZDI3RBC2 (*L. biglobosa brassicae*), RHZDI3OSR3.1 (Uncultured Mortierella) and RFMMN9 (*Penicillum sp.*) all belong to taxonomic groupings with a record of functional analysis in plants. By comparison, no plant based functional research has been presented to date for members of the Geomyces genus RFOSR1 (*Geomyces pannorum*).

The Phomopsis genus includes endophytic and pathogenic species, including *P. viticola* the causative agent of dead arm, a disease of grape vines (Uecker, 1988,

Úrbez-Torres et al., 2013). Members of the genera have not been documented as pathogens of OSR, however the results presented here indicate that Phomopsis species can have negative impacts on OSR seedling biomass, identifying this genus as potentially pathogenic.

Penicillium species are also known to be pathogenic to a variety of plants including orchard crops such as pear and apple (Franke-Whittle et al., 2015). However, Penicillium species have not been identified as pathogenic to OSR with the results presented in this study indicating their pathogenic potential in OSR for the first time.

L. biglobosa brassicae in conjunction with its relative *L. maculans* are major pathogens of OSR being the causative agents of blackleg disease (AHDB, 2021, Grandaubert et al., 2014). However, compared to its relative *L. maculans, L. biglobosa brassicae* has been identified as a less aggressive pathogen (Grandaubert et al., 2014). By contrast, *L. biglobosa brassicae* isolates have also been identified as a potentially beneficial endophytes of OSR, having been shown to increase OSR biomass (Zhang et al., 2014). This indicates that strain differences between *L. biglobosa brassicae* isolates may determine function. In addition to *L. biglobosa brassicae*, Zhang et al. (2014) also identified Fusarium, Alternaria and Aspergillus isolates which conferred beneficial traits to OSR indicating that fungal taxa typically known as plant pathogens may also be beneficial endophytes of OSR. Indeed, Fusarium sp. OTUs identified in Hilton et al., (2021) were found to be positively correlated with OSR yields.

In contrast, to the aforementioned taxa with known deleterious effects on plant biomass and health, the Mortierella genus contains many beneficial species which are widely documented as plant growth promoting fungi. They are known to confer a range of beneficial traits such as, pathogen biocontrol, abiotic stress tolerance, phytohormone modulation and biofertilisation (Ozimek and Hanaka, 2021). Therefore, the significant reduction in OSR seedling biomass related to inoculation with RHZDI3OSR3.1, an 'Uncultured Mortierella' isolate, indicates that certain Mortierella species may be deleterious associates in OSR. This is especially true given that the related isolate RFRBC4 showed no significant impact on OSR biomass. These isolates were matched to the highly abundant and distributed 'Unidentified Mortierella' OTU3 in the UK OSR rhizosphere mycobiome dataset. Given the wide distribution of Mortierella taxa in the OSR rhizosphere mycobiome, their documentation as beneficial associates of many plant species and the deleterious

impact identified in this study, Mortierella taxa can be seen as particularly important taxa for further analysis in OSR.

In contrast to taxa with well documented functional analysis in plant species, there is no research regarding the function of Geomyces in the context of plants. The Geomyces are temperate soil fungi with a global distribution (Hayes, 2012) having been found in agriculture and artic soils (Arenz et al., 2006, Domsch et al., 1980). The Geomyces genus is composed of five species including the causative agent of the major bat disease White Nose Syndrome (*G. destructans*). However, the most commonly identified Geomyces species is *G. pannorum* (Hayes, 2012). Most research focused on *G. pannorum* has examined its production of bioactive metabolites and applications in industrial pollution bioremediation (Cosgrove et al., 2007, Fenice et al., 1997). Therefore, further investigation of this isolate to understand its potentially deleterious impact upon OSR and plants in general is of particular interest due to its widespread distribution in global soils and the lack of functional analysis in plants.

In comparison to the aforementioned isolates, a number of isolates which were aligned to taxa previously shown to be corelated with OSR yields, were found to have no significant effect on OSR biomass in this study. Isolate RFOSR8 (*Tetracladium maxilliforme*) was matched to OTU19, an OTU widely distributed in OSR root mycobiomes and positively correlated with yield (Hilton et al., 2021). Tetracladium species are known as aquatic hyphomycetes but have also been detected as endophytes in roots of a variety of crop species (Franke-Whittle et al., 2015, Selosse et al., 2008). In addition, Tetracladium species have also been shown to be metabolically active in the OSR rhizosphere, actively assimilating plant derived carbon (Gkarmiria et al., 2017). In this study, *T. maxilleforme* was not found to have a significant impact on OSR seedling biomass suggesting another mechanism for the positive association between OSR yield and *T. maxilleforme* abundance identified in Hilton et al. (2021). As previously discussed in section 3.5.2, strain level differences between fungi can also affect functional capabilities and therefore may also factor into differences in functional capabilities observed between studies.

3.5.4 Directions for further investigation

The research presented here provides the first steps towards exploring the functional diversity of OSR associated rhizosphere fungi in the UK and provides a number of candidate isolates for further investigation. This is required to unravel the full functional potential of these isolates and examine causative relationships between the significant correlations to OSR yield outcomes identified by Hilton et al. (2021) and isolate impacts on OSR biomass identified in this study.

Firstly, full genome sequencing of isolates using emerging technologies such as Oxford Nanopore MinIon™ would allow the identification of functional genes within the housekeeping or accessory genomes from which functional capabilities of isolates can be predicted (Irinyi et al., 2017, Jain et al., 2016, McCarthy and Fitzpatrick, 2019). Full genome sequencing of fungi is particularly important given the smaller number of full genome sequences available for fungi compared to bacteria (Hibbett et al., 2013). In addition, full genome sequencing would allow discrimination of isolates at the strain level and may aid in resolving differences between isolates matched to the same OTU in this study but with different impacts on plant biomass such as isolates RFRBC4 and RHZDI3OSR3.1 matched to OTU3 (Jain et al., 2016).

As previously discussed, sequencing of isolates cannot be used alone to determine their functional potential with plants and plant-microbe interaction studies are required to complement genomic analysis (Peay, 2014). The plant-microbe co-incubations studies presented here provide an initial insight into the functional capabilities of several UK OSR associated fungal isolates, with several isolates found to impact OSR seedling biomass. However, further analysis is required to uncover the underlying functional processes and interactions between these isolates and OSR.

Firstly, satisfaction of Koch's postulates by reisolating isolates from infected OSR plants in axenic co-incubation studies would provide a firm basis for a causal relationship between the presence of isolates and OSR biomass outcomes (Bhunjun et al., 2021). Subsequently, further interrogation of axenic plant-fungi interaction studies using various techniques such as; microscopy of root tissues to identify fungal symbiotic or invasion structures (Hardham, 2011) and multi-omics technologies such as transcriptomics (Vangelisti et al., 2018) and proteomics (Jain et al., 2021) to

understand fungal and plant metabolic processes activated during interactions in root tissues.

In addition to incorporation of other analyse to plant-fungi co-incubation studies, alteration of study parameters would also provide insight into how fungi/plant growth stages may affect interactions. Alteration of plant parameters such as extension of co-incubation study duration to the silique formation to pod shatter growth stages would allow assessment of fungal isolate impact upon OSR yield as was conducted by Hilton et al. (2013). At the opposite end of the OSR lifecycle, infection of OSR seeds with fungal isolates would allow analysis of germination inhibition or promotion capabilities of fungal isolates. Alteration of fungal parameters, such as the specific fungal life stage used for inoculation i.e., vegetative mycelia or conidia, would also allow investigation of how potential interactions are established between fungi and the plant host.

4 Immune hormone deficient *Arabidopsis thaliana* root mycobiomes and their soil legacy.

4.1 Introduction

Root inhabiting fungi are recognised as important determiners of plant health with a multitude of beneficial, commensal, and pathogenic taxa competing to infect and inhabit root tissues (Berendsen et al., 2012, Chen et al., 2018, White et al., 2019). As such, they are regarded as key targets for use in crop protection and enhancement strategies (Busby et al., 2017, Pozo et al., 2021, White et al., 2019). However, there is a lack of a systemic understanding of how fungi (and the root microbiome in general) assembles in response to each other, the plant host and how this is influenced by interactions with the environment.

Of particular interest is how root microbiomes assemble in response to plant hormones (or 'phytohormones') produced and modulated by the plant host and/or associated microbiota (Berger et al., 2020, Großkinsky et al., 2016, Pieterse et al., 2012). These phytohormones form complex regulatory networks which regulate plant growth and development (auxins, cytokinins, gibberellins and brassinosteroids), and physiological processes including immune responses to biotic stressors such as microbial pathogen infection (abscisic acid, ethylene, jasmonic acid and salicylic acid) and responses to abiotic stressors such as drought (abscisic acid) (Großkinsky et al., 2016).

Phytohormones involved in regulating the plant immune system are particularly interesting given that plants are sessile organisms which encounter a diverse and complex range of pathogenic, beneficial and commensal microorganisms in their direct environment (Pieterse et al., 2012). Indeed, plants must discern between beneficial and pathogenic microorganisms which seek to inhabit its root tissues (Pieterse et al., 2012). A range of phytohormones are involved in regulating the plant immune system including Abscisic (ABA), Jasmonic (JA) and Salicylic Acid (SA). In addition to immune system regulation, ABA, JA and SA regulate plant growth, germination and senescence (Bari and Jones, 2009). These phytohormones are known to interact with, and counter regulate each other in response to tissue colonisation by

beneficial and pathogenic microbes (Bari and Jones, 2009). Also, certain beneficial (Yu et al., 2019) and pathogenic (El Oirdi et al., 2011) taxa are known to interact and interfere with these hormone pathways in order to successfully colonise root tissues. In particular, SA is involved in immune responses to biotrophic pathogens and the formation of systemic acquired resistance (Grant and Lamb, 2006). Whereas JA is involved in the immune response to necrotrophic pathogens (Bari and Jones, 2009). Less research has been presented on ABA; however, increasing interest is being shown in investigating its role in plant immune responses as it has been shown to modulate responses to pathogens in concert with JA and SA (Fan et al., 2009, Sánchez-Vallet et al., 2012).

Therefore, these phytohormones can be seen as key players in microbial infection and colonisation processes in root tissues. Subsequently they may represent powerful tools for host mediated microbiome manipulation. However as previously discussed, most research has focused on how these hormones are regulated in planta in response to known pathogens or beneficial taxa. Since plants interact with a multitude of different microbial taxa simultaneously, further investigation into how microbiomes assemble in response to these hormones is essential before their use in cropping strategies can be considered. This is particularly important when concerning rhizosphere fungi, given that they form a range of deleterious (pathogenic) and beneficial (symbiotic) process within root tissues, sometimes through immune phytohormone regulation (Berendsen et al., 2012, Chen et al., 2018, Pieterse et al., 2012, White et al., 2019). In addition, our understanding of the relevance of rhizosphere fungal communities as a whole to plant health and microbiome structure is less advanced than that of bacteria (Pozo et al., 2021). Therefore, investigation of rhizosphere fungal microbiome assembly in relation to immune phytohormones is particularly important.

A small body of research exists regarding the formation of bacterial microbiomes in response to JA and/or SA in *Arabidopsis thaliana* and *Triticum aestivum* (Carvalhais et al., 2013, Carvalhais et al., 2015, Lebeis et al., 2015, Liu et al., 2017). In *A. thaliana,* bacterial microbiome assembly in response to activation and disruption of JA biosynthesis pathways has been investigated (Carvalhais et al., 2013, Carvalhais et al., 2015). From this research, activation of JA biosynthesis pathways with exogenous application of methyl jasmonate was found to alter bacterial

community assembly, with enrichment of plant defence related taxa reported (Carvalhais et al., 2013). Also, genotypes with disrupted jasmonate biosynthesis pathways showed formation of rhizosphere bacterial communities distinct from the wild-type genotype (Carvalhais et al., 2015). In *T. aestivum,* activation of JA biosynthesis through exogenous application of methyl jasmonate resulted in a decrease in root community diversity. However, an increase in bacterial taxa associated with plant growth promotion, pathogen suppression and nutrient acquisition were identified (Liu et al., 2017). Regarding SA, impaired SA biosynthesis in *A. thaliana* altered the colonisation of the bacterial root microbiome at the phylum and family taxonomic levels (Lebeis et al., 2015). In addition, it has been shown that exogenous application of SA can influence the formation of bulk soil microbiota, indicating that plant hormones may play a role in soil microbial assembly (Badri et al., 2013). Therefore, there is evidence that JA and SA are involved in the assembly of some root and rhizosphere microbial communities.

To date, one publication regarding the assembly of fungal communities in relation to a defence phytohormone has been published (Manzotti et al., 2020). In this study, a *Solanum lycopersicum* genotype with impaired JA biosynthesis was found to have a significantly more diverse fungal root community compared to the background genotype. However, no differences in overall community composition and OTU relative abundances were identified. No research has been conducted into how other immune phytohormones such as SA and ABA may be involved in root mycobiome assembly. Unlike SA, there is also no research on how ABA may influence the assembly of any microbial kingdom in any case. In addition, no research into how these dynamics relate to host plant biomass or their legacy upon the soil microbiota has been presented. This leaves key knowledge gaps in our understanding of how fungal root communities assemble in relation to immune phytohormones and what this means for the plant host, soil microbiome legacy and the use of immune phytohormones in cropping strategies.

4.2 Aims

In this chapter it was hypothesised that immune phytohormone mutant and wild-type *A. thaliana* genotypes could be used to elucidate the relationship between fungal root microbiome assembly and plant immune phytohormones and whether 'deleterious' and 'beneficial' root mycobiomes formed. To these ends, the molecular characterisation of root mycobiome community assemblies in three immune phytohormone mutant *A. thaliana* genotypes were carried out to:

1. Determine how root mycobiomes assemble in two immune impaired genotypes of *A. thaliana* deficient in either Abscisic and Salicylic acid biosynthesis, and one *A. thaliana* genotype hypersensitive to Abscisic acid.

2. Determine whether immune deficient genotypes assemble 'deleterious' microbiomes compared to wild-type through examination of functional guilds and the relationship between mycobiome assembly and plant biomass.

3. Determine whether transplantation of wild-type and immune deficient genotype conditioned soils can have a beneficial or compounding legacy effect on subsequent plant biomass and root mycobiome assembly.

4.3 Materials and Methods

4.3.1 Experimental design

This study was split into two main experimental phases to address the experimental aims identified in section 4.2. The first experimental phase (Phase I) concerned the assessment of root mycobiome assembly in response to the four *A. thaliana* genotypes utilised in this study (wild-type Col0, Sid2, Aao3 & Aba). The three mutant genotypes were either deficient in the biosynthesis of a key immune phytohormone (SA (Sid2) & ABA (Aao3)) or hypersensitive to an immune phytohormone (ABA (Aba)). This phase of experimentation aimed to observe whether root mycobiomes assembled differently in these three immune mutated genotypes and whether immune deficient genotypes assembled a 'deleterious' root mycobiome. The second experimental phase (Phase II) concerned examination of legacy effects of immune deficient *A. thaliana* upon soil mycobiomes. Soil conditioned by the growth of either wild-type Col0 or Sid2 *A. thaliana* was collected from the Phase I experiment. This soil was then used as a microbial inoculum in sterile growth media used for growing Col0 and Sid2 *A. thaliana*. This enabled the examination of whether transplantation of wild-type Col0 and immune deficient Sid2 conditioned soils can have a beneficial or compounding legacy effect on subsequent plant root mycobiome assembly and biomass. Together, these two phases of experimentation enabled examination of plant immune hormone – root mycobiome dynamics and whether there were subsequent legacy effects upon conditioned soils.

4.3.1.1 *A. thaliana* genotypes and experimental soil

Wild-type Col0 (Columbia ecotype) and three mutant *A. thaliana* genotypes, Sid2, Aao3 and Aba, were used in this study. Col0 is the background genotype for the three mutant genotypes. The Sid2 genotype is a KO mutant of isochorismate synthase which results in disruption of SA biosynthesis and SA deficiency in this genotype (Nawrath and Métraux, 1999). The Aao3 genotype is a KO of Arabidopsis Aldehyde III, the penultimate enzyme in the biosynthesis of stress activated ABA which results in disruption of ABA biosynthesis and ABA deficiency in this genotype (de Torres Zabala et al., 2015). Finally, the Aba genotype has three mutations in protein phosphatase 2C, rendering this genotype hypersensitive to ABA (de Torres-Zabala et al., 2007).

Experimental soil was a Wick series sandy loam soil (16.3% clay, 16.6% silt and 67.1% sand (Whitfield, 1974)) sampled from the permanent grass field margin of Cottage Field West, University of Warwick Wellesbourne Campus, UK on the 20th of July 2020. This soil has a carbon content of 0.9 % and a pH of 6.8 (Whitfield, 1974). Soil was sampled at a depth of 0 – 20 cm and sieved to a particle size of 3 mm to remove plant root tissues and debris. Processed soil was subsequently store at 5 °C until required for use in experimentation.

4.3.1.2 Phase I experimental design

This experimental phase was designed to address whether Aba, Aao3 and Sid2 *A. thaliana* immune hormone mutants assembled significantly different root mycobiomes to wild-type Col0 *A. thaliana*. In addition, this experimental phase provided Col0 and Sid2 conditioned soils for analysis of soil microbial legacy.

Sieved Wellesbourne soil was transferred to modular plant pot trays (5 x 8 pots, 4 cm diameter) to the inner lip level of each pot. These pots were then separated across five holding trays following a randomised design to control for position influences. Col0, Sid2, Aba and Aao3 *A. thaliana* seeds were then sown in a randomised design (see section 4.3.2). Plants were then maintained for 12 weeks (see section 4.3.2) until harvesting of root and soil tissues for DNA extraction (see 4.3.3) and collection of conditioned Col0 and Sid2 soils for the Phase II experiment (see section 4.3.1.3)

4.3.1.3 Phase II experimental design

After plant harvesting for DNA extraction in phase I (see section 4.3.1.3 & 4.3.3) pots containing remaining bulk soil from wild-type Col0 and SA deficient Sid2 genotype treatments were combined (by genotype) to form a microbial inoculant each for Col0 and Sid2 conditioned soil. This allowed examination of whether any legacy effects of microbiome transfer upon subsequent plants occurred i.e., a compounding effect (Col0 inoculant – Col0 seeds / Sid2 inoculant – Sid2 seeds), a 'rescuing' effect for immune deficient genotypes (Col0 inoculant – Sid2 seeds), or a deleterious effect for the wild-type genotype (Sid2 inoculant – Col0 seeds).

These inoculants were prepared using a modified version of the soil microbiome transfer inoculant preparation protocol presented in (Howard et al., 2017). Firstly, the top 1 cm of bulk soil in pots were disposed of to remove any influence of

biological soil crust communities observed in pots. The remaining bulk soil was then combined (by genotype treatment) into sterilised 5000 ml glass beakers and gently homogenised. From this, each genotype combined bulk soil was then transferred to a second sterile 5000 ml glass beaker to the 1000 ml mark. To this a 50/50 v/v mix of sterilised sharp sand and expanded clay was added to the 5000 ml mark (This sand clay mixture was treated with Hoagland's Solution to a concentration of 1x Hoagland's Solution per 1000 ml of sand clay mixture). The addition of nutrient solution treated sterilised sand/clay mix was carried out to allow transfer of a representative microbiome whilst masking any differences in soil nutrient profiles between genotypes (**Appendix Table S1**). Each prepared soil inoculant was then transferred to modular plant pot trays (5 x 8 pots, 4 cm diameter) to the inner lip level of each pot. These pots were then separated across two holding trays following a randomised design to control for position influences. Col0 and Sid2 *A. thaliana* seeds were then sown in a randomised design (see section 4.3.2).

4.3.2 Preparation and maintenance of plants

A. thaliana seeds were stratified in sterile deionised water for 72 hrs at 5 ºC. For Phase I: Col0, Aao3, Aba and Sid2 stratified seeds were sewn into sieved Wellesbourne soil following a randomised design (In total 30 pots were sown for each genotype). For Phase II: Col0 and Sid2 stratified seeds were sewn into Col0 or Sid2 conditioned soil inoculant following a randomised design (In total 20 pots were sown for each treatment condition). Between 10-15 seeds were sewn at five equidistant points in each pot using a sterile pipettor. Sown pots were then moved into a Sanyo Versatile Environmental Test Chamber MLR-350 (Sanyo, Moriguchi, Japan) for a period of; twelve weeks (Phase I experiment) and four weeks (Phase II experiment). Growth conditions were as follows; cycle = 14 hrs day - 10 hrs night, temperature = 21 ºC constant day/night and light intensity = 120 μE. Plant pots were weighed three times per week to track water loss and sterile deionised water added as necessary to maintain 33 % water holding capacity. After watering, trays were rotated 180 º and rotated up and down growth cabinet shelves to minimise any cabinet position effects. At the two-week timepoint pots were thinned to maximum five plants per pot. Plants were also checked for the presence of reproductive tissues, with any flower bolt

formation being halted by physical removal of emerging flower bolts using sterilise scissors.

4.3.3 Root and soil sampling for DNA extraction

For the Phase I experiment, between 5 and 10 plants were harvested from two randomly assigned pot modules and combined to create a single sample. For each genotype ten samples were created for DNA extraction and microbiome sequencing. For the Phase II experiment, only Col0 seeds germinated. Plants were harvested from a single pot to produce a single sample. For Col0 – Col0 seven samples were collected and for Sid2 – Col0 nine samples were collected. For each sample between one and five plants were harvested.

For both experimental Phases, root systems were harvested from each plant by carefully removing plants from soil, brushing off loosely adhering soil with sterile tweezers and cutting root systems from plants at the root shoot interface. Root systems from replicate plants were combined into a sterile 2 ml universal tube containing sterile deionised water and inverted 30 times. This was then repeated for two rounds. Washed root systems were then blotted dry with a sterile cotton bud and stored at -80 ºC until DNA extraction. After collection of root systems leaf number was documented for each harvested plant to provide a mean leaf number for each combined sample. Plant shoot systems were retained for analysis of shoot biomass. Shoot systems were grouped by sample and placed into paper bags and transferred to a drying oven for four days at 70 °C. Dried plant material was then weighed for each sample.

4.3.4 Next Generation amplicon sequencing

4.3.4.1 DNA sample preparation and sequencing

DNA extraction was carried out using a FastDNA™ kit for Soil (MP Biomedicals, Irvine, USA) with mechanical disruption of cells carried out using a FastPrep-24™ homogeniser (MP Biomedicals, Irvine, USA) for 2 x 30 seconds at a speed of 6 m s^{-1}. Extracted DNA was quantified with an Invitrogen Qubit® fluorometer 2.0 (Invitrogen brand, Thermo Fisher Scientific, Waltham, USA) using the broad range DNA assay. DNA samples were then diluted to a working concentration between 1 – 10 ng/µl. Amplification of the ITS2 rRNA region was carried out using the fITS1 (Ihrmark et al., 2012) and rITS4 (White et al., 1990) fungal primers. These primers were modified at the 5′ end with adapters from a dual-index sequencing

strategy (Kozich et al., 2013). PCR reactions were performed in a reaction volume of 25 µl, containing Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs®, Ipswich, USA) and 0.5 µM of each primer. PCR conditions were as follows: 95 ºC for 2 min, 30 cycles of 95 ºC for 30 s, 52 ºC for 30 s, 72 ºC for 2 min and then final extension of 72 ºC for 10 min. Amplified DNA samples were then purified using magnetic purification beads. The Illumina® Nextera® Index PCR system (Illumina®, San Diego, USA) was then utilised to tag each samples' sequences for demultiplexing post sequencing. Index PCR reactions were performed in a reaction volume of 25 µl, containing Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs®, Ipswich, USA) and 0.5 µM of each primer. PCR conditions were as follows: 95 °C for 3 min, 8 cycles of 98 °C for 20 s, 55 °C for 15 s, 72 °C for 15 s and 72 °C for 5 min. Following index tagging of sequences, samples were normalised using a SequalPrep™ Normalisation Kit (Invitrogen brand, Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. Samples were subsequently pooled and concentrated to 4 nM using a SpeedVac™ vacuum concentrator. Sequencing was carried out using a PE 300 bp run on an Illumina® MiSeq™ (Illumina®, San Diego, USA) with dual indexing at the University of Warwick Genomics Facility, Coventry, UK.

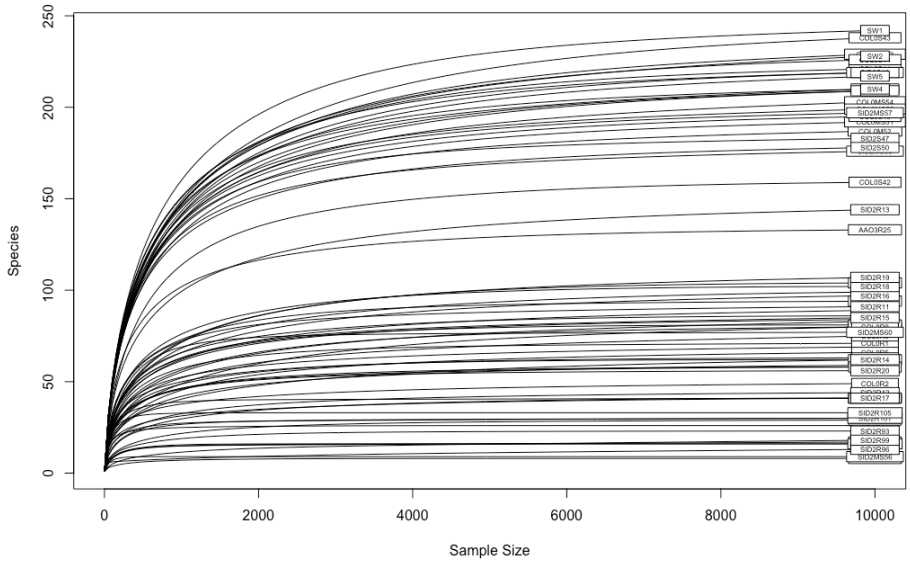

Figure 4.1 Rarefaction curve for sequenced samples. *Samples were rarefied to 10,000 reads with 67 samples being retained. X-axis is representative of number of reads in samples with the y-axis representative of the number of unique species. N = 67.*

4.3.4.2 Post sequencing processing

Raw sequences were provided demultiplexed by the University of Warwick Genomics Facility. Raw sequences were processed using the Qiime2 v.2021.2 command line interface to produce a final ASV table (Bolyen et al., 2019). ITS primers were removed using q2-cutadapt. Sequence truncation, denoising and merging of paired ends was then carried out using DADA2 (Callahan et al., 2016). Due to variation in ITS sequence lengths across fungal taxa, sequences were truncated based upon base quality score using a Q value of 10. Taxonomy was then assigned in Qiime2 using the dynamic UNITE database v8.0 (Kõljalg et al., 2013, UNITE-Community, 2019). From this dataset a total of 6,135,175 reads were obtained with 1,728 ASVs across 80 samples. The assigned ASV data was then exported as an ASV table for rarefaction using the vegan programme in R (Jari Oksanen 2019, McMurdie and Holmes, 2013, R Core Team, 2013, R Core Team, 2018). This ASV table was rarefied to 10,000 reads (**Figure 4.1**). This resulted in 670,000 reads (10.92 %) across 67 samples being retained, with community richness plateauing for all samples at this rarefaction level (**Figure 4.1**). This rarefied ASV read table was subsequently analysed in R for bioinformatic and statistical analysis of fungal communities (section 4.3.5).

4.3.5 Bioinformatic analysis

4.3.5.1 Bioinformatic and statistical analyses

Bioinformatic analyses were carried out using R software (R Core Team, 2013) with a range of R packages used to analyse ASV tables in the RStudio software environment (RStudio Team, 2015). Unless otherwise stated, statistical analyses were carried out using the R stats package (R Core Team, 2018). Graphs were produced using the ggplot2 package (Wickham, 2016) with the following add-on packages; ggpubr (Kassambara, 2018), ggrepel (Kamil Slowikowski, 2019), ggalt (Bob Rudis, 2017), ggforce (Lin Pedersen, 2019), cowplot (O. Wilke, 2019) and ggfortify (Tang, 2016, Yuan Tang, 2016) unless otherwise stated. Normality of data assessed using the Shapiro-Wilk normality test where appropriate. Unless otherwise stated in sections 4.3.5.2 and 4.3.5.3, data were assessed using the Kruskal-Wallis test with Dunn's Test post hoc analysis and Wilcoxon Rank Sum for non-parametric data analysis in R (R Core Team, 2013, R Core Team, 2018).

4.3.5.2 Analysis of microbial community sequence data

Microbial community assembly of root and soil/soil inoculant samples from experimental phases I and II were analysed using a number of microbial ecology analysis packages in the R studio environment (R Core Team, 2013, R Core Team, 2018). Analysis was carried out using the phyloseq v1.34.0 (McMurdie and Holmes, 2013) and vegan v2.5.7 (Jari Oksanen 2019) microbial ecology analysis packages unless stated otherwise. These analyses allowed the determination of whether immune hormone mutant *A. thaliana* genotypes assembled 'deleterious' microbiomes in phase I and whether there was a legacy effect of these deleterious microbiomes upon subsequent plants in phase II.

Community $\beta-$diversity was assessed using NMDS ordination of community $\beta-$dissimilarity matrices with subsequent PERMANOVA and ANOSIM analysis. PERMANOVA analysis allows determination of significant differences in $\beta-$dissimilarity matrix centroids for each *A. thaliana* genotype (Phase I) or soil/soil inoculant and treatment (Phase II). ANOSIM analysis provides a value of community dissimilarity (R^2) and corresponding level of significance (p). R^2 values range from -1 to +1, with more positive values indicating dissimilarity due to differences between groups and negative values indicating a higher level of variation within groups than between groups.

Community alpha $\alpha-$diversity was determined using the Fisher's Alpha method on ASV read tables with significance tested using the Kruskal-Wallis test with a Dunn's Test post-hoc analysis (from the FSA R package, v0.8.32 (Ogle et al., 2021)).

The aforementioned analyses concerned analysis of microbial community assemblies at the ASV level. Community composition at lower taxonomic levels (Phylum to Genus) was assessed using comparison of taxa relative abundancies between genotypes in phase I and soil/soil inoculants, and treatments in phase II. Significance differences between comparisons were identified using the Kruskal-Wallis test with a Dunn's Test post-hoc analysis.

4.3.5.3 Mycobiome assembly and plant biomass

In order to determine if certain fungal taxa had a significant effect upon *A. thaliana* biomass in phase I and II of the experiment, correlation analysis between taxa

and biomass data was conducted. This enabled examination of whether immune deficient *A. thaliana* genotypes assembled 'deleterious' microbiomes in phase I and whether there was a legacy effect of these deleterious microbiomes upon subsequent plants in phase II.

Correlation analysis was carried out using the spearman's correlation function in R with the Bonferroni multiple comparison correction was carried out on ASV read tables versus dry shoot weight and average leaf number meta data.

4.4 Results

4.4.1 Phase I: Determination of root mycobiome assembly in wild-type and immune phytohormone mutant *A. thaliana* genotypes

4.4.1.1 Community assembly at the ASV level was not affected by *A. thaliana* immune phytohormone mutant genotype.

ASV level analysis of community α and β diversity indices indicated that there were no significant differences in the assembly of root mycobiomes between wild-type Col0 and mutant Sid2, Aao3 and Aba *A. thaliana* genotypes. Distinct clustering of root mycobiomes by genotype was not observed in NMDS ordination of Bray-Curtis $\beta-$dissimilarity matrices (**Figure 4.2A**). In addition, no significant differences between matrix centroids were identified by PERMANOVA analysis (**Table 4.2**), indicating that there were no significant differences in overall community composition between genotypes at the ASV level. Fisher's α diversity analysis also showed no significant differences in overall community diversity amongst genotypes (**Figure 4.2B)**.

Table 4.1 PERMANOVA analysis output for comparison of Col0, Sid2, Aba and Aao3 genotype β-dissimilarity matrix centroids.

	df	sum of squares	mean squares	f. model	r^2	p-value
genotype	3	0.9950	0.33168	1.245	0.11409	0.062 (ns)
residuals	29	7.7261	0.26642		0.88591	
total	32	8.7212			1.00000	

However, at lower taxonomic resolution, significant differences ($p < 0.05$) in the relative abundances of the Basidiomycota (Phylum) and Sordariales (Order) between wild-type Col0 and ABA mutant genotypes were identified (**Figure 4.3 & 4.4**). Basidiomycota were found to be significantly ($p < 0.05$) enriched in genotypes Aao3 (mean RAB = 5.042 %) and Aba (mean RAB = 4.167 %) compared to wild-type Col0 (mean RAB = 0.317 %) and at the Order level, the Sordariales were found to be

significantly ($p < 0.05$) enriched in Aao3 (mean RAB = 8.907 %) compared to wild-type Col0 (mean RAB = 1.298 %) (**Figure 4.3 & 4.4**).

4.4.1.2 *A. thaliana* immune hormone mutant genotypes did not assemble significantly different communities of functionally significant fungal guilds.

Functional inference using FunGuild was carried out to assess the assembly of functionally significant fungal guilds between immune hormone mutant genotypes and wild-type *A. thaliana*. No significant differences were identified between genotypes in the relative abundances of plant pathogens, fungal endophytes, and mycorrhizas (**Figure 4.5**).

4.4.1.3 No correlation between fungal taxa and plant biomass was identified in immune hormone mutant and wild-type *A. thaliana* genotypes.

Assessment of mycobiome function in relation to plant biomass was also carried out to determine if beneficial or deleterious mycobiomes were assembled in the different genotypes assessed in this study. No significant difference in shoot biomass was identified between immune hormone mutant and wild-type *A. thaliana* genotypes (**Figure 4.6A**). However, the Aba mutant genotype was found to have significantly ($p < 0.05$) lower mean leaf number than wild-type Col0 (**Figure 4.6B**). Subsequently, Spearman's correlation analysis between ASV relative abundances and the plant biomass data did not identify taxa positively or negatively correlated with plant dry biomass and leaf number. This indicates that fungi within the root mycobiome of these four *A. thaliana* genotypes did not contribute to host biomass or the differences observed in leaf number between the Aba and Col0 genotypes.

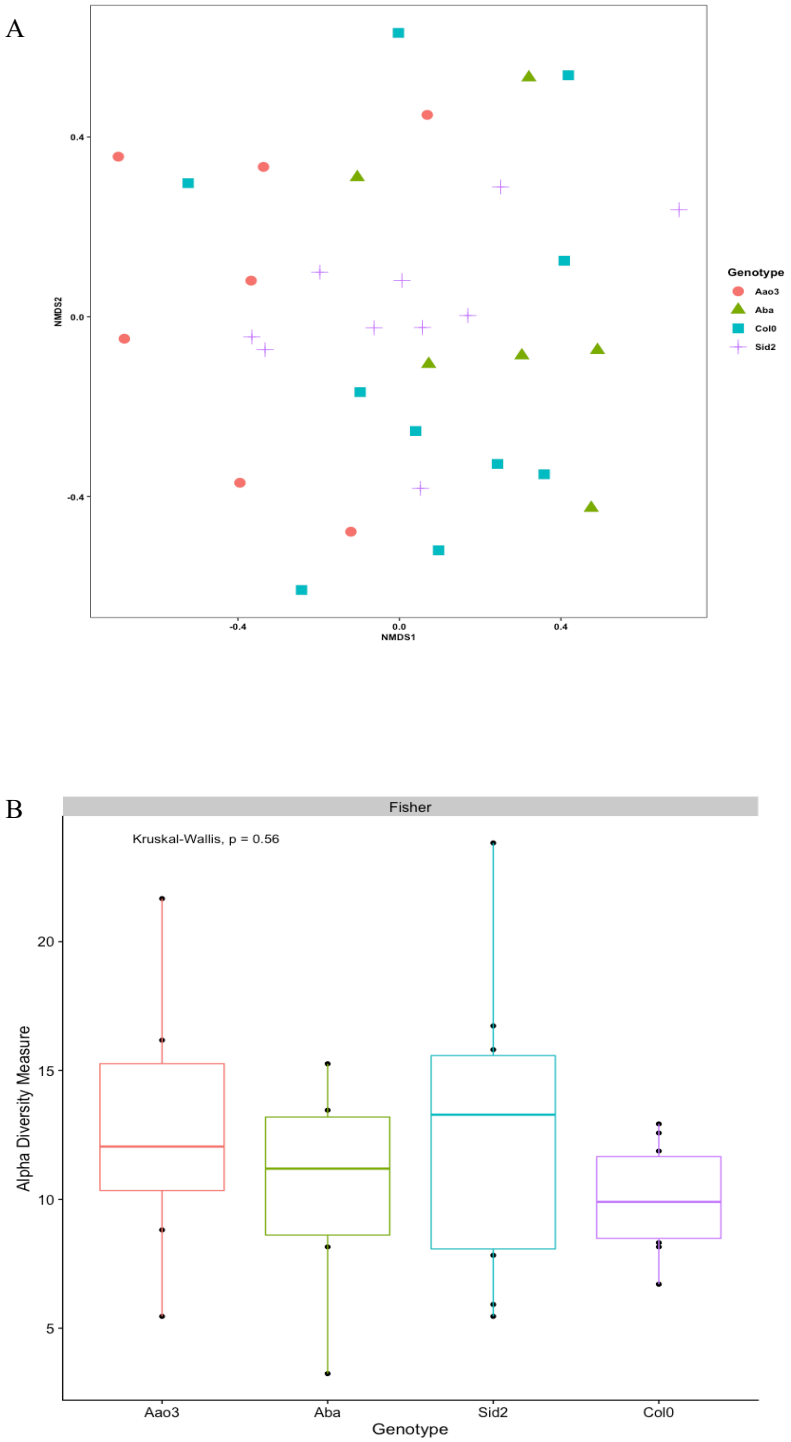

Figure 4.2 Community diversity metrics of A. thaliana genotype fungal root microbiome. *(**A**) NDMS ordination of genotype community* $\beta - dissimilarity$ *matrices based on Bray Curtis dissimilarity. Statistical testing carried out using ADONIS analysis of dissimilarity matrix centroids with 999 permutations. (**B**) Alpha diversity analysis using* $Fisher's\ \alpha$ *diversity analysis. Statistical testing carried out using the Kruskal-Wallis non-parametric analysis for multiple groups with test p-value listed in top left corner of graph. Sample number for each genotype; Aao3 = 7, Aba = 6, Sid2 = 10, Col0 = 10.*

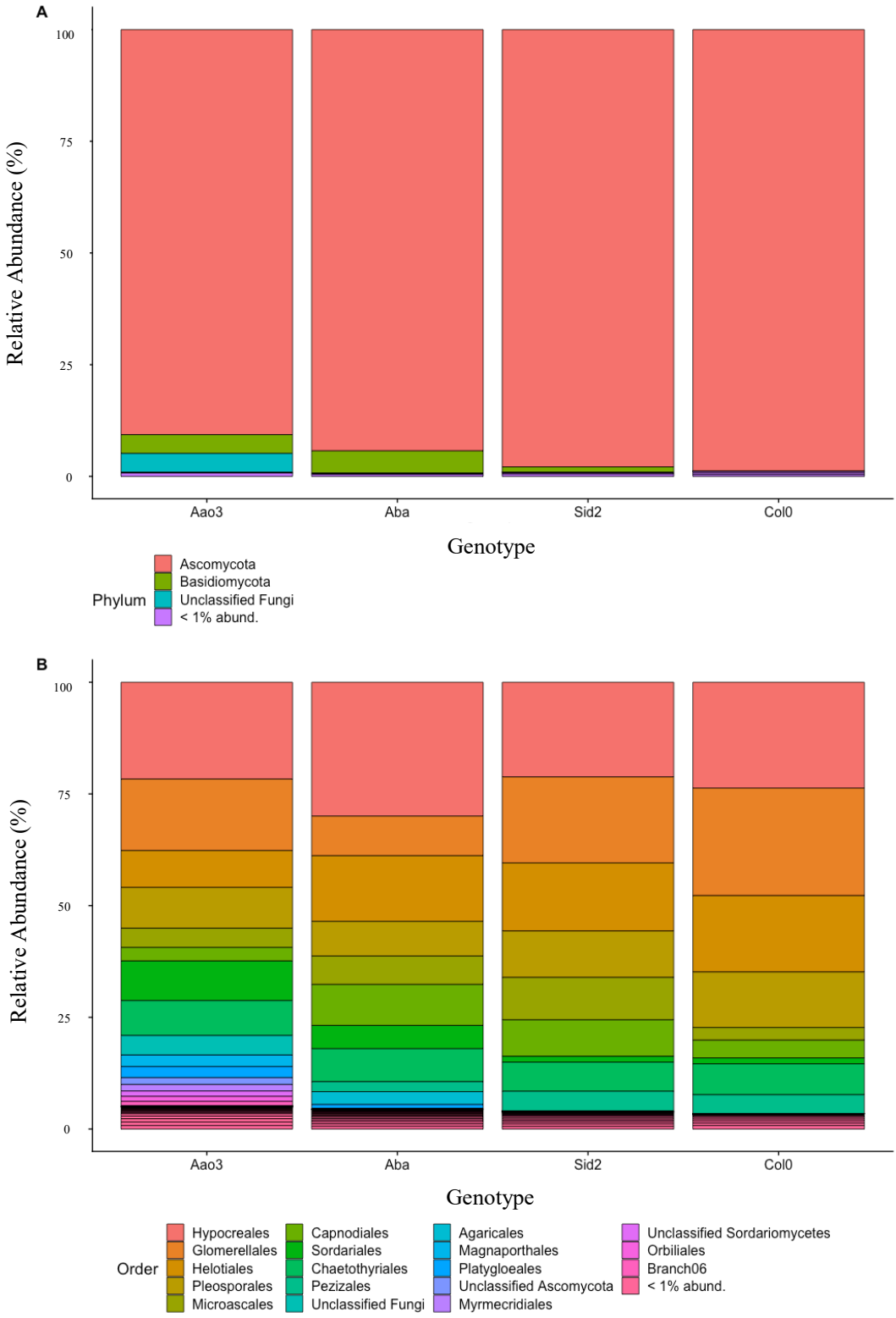

Figure 4.3 Stacked Bar plot of community relative abundances. *Relative abundance of genotypes grouped at Phylum (**A**) and Order (**B**) taxonomic levels. Sample number for each genotype; Aao3 = 7, Aba = 6, Sid2 = 10, Col0 = 10.*

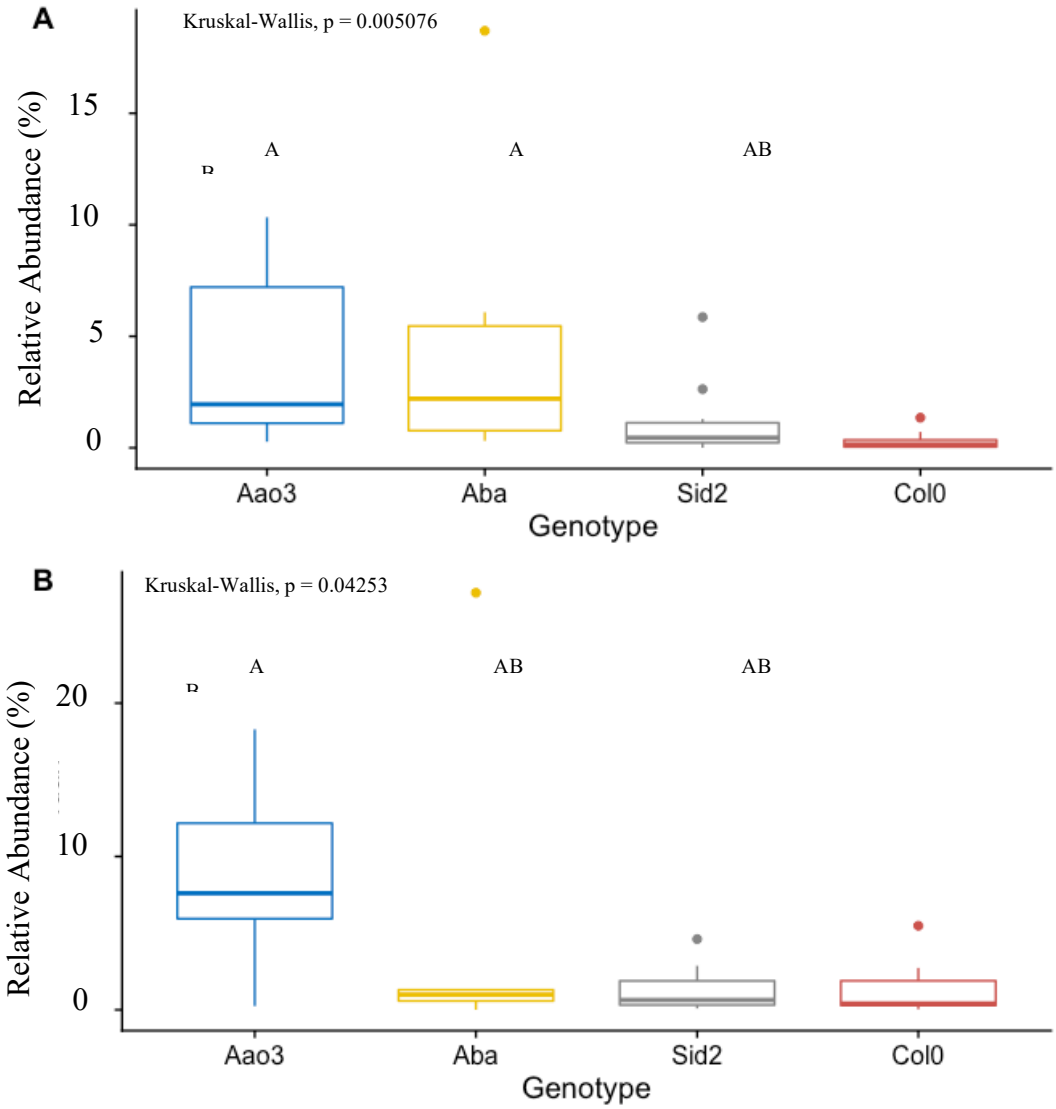

Figure 4.4 Boxplot of relative abundances of Phylum and Order taxa with significant differences between A. thaliana genotypes*. (A) Relative abundances of the Basidiomycota and (**B**) relative abundances of the Sordariales. Statistical testing carried out using the Kruskal-Wallis non-parametric analysis for multiple groups with test p-value listed in top left corner of graph. Dunn's Test post-hoc analysis with Bonferroni multiple comparison correction was employed to identify significantly different comparisons. Letters are indicative of significantly different ($p < 0.05$) groups (different letters = significantly different groups). Whiskers are representative of inter quartile range with dots representative of outliers based on interquartile range. Sample number for each genotype; Aao3 = 7, Aba = 6, Sid2 = 10, Col0 = 10.*

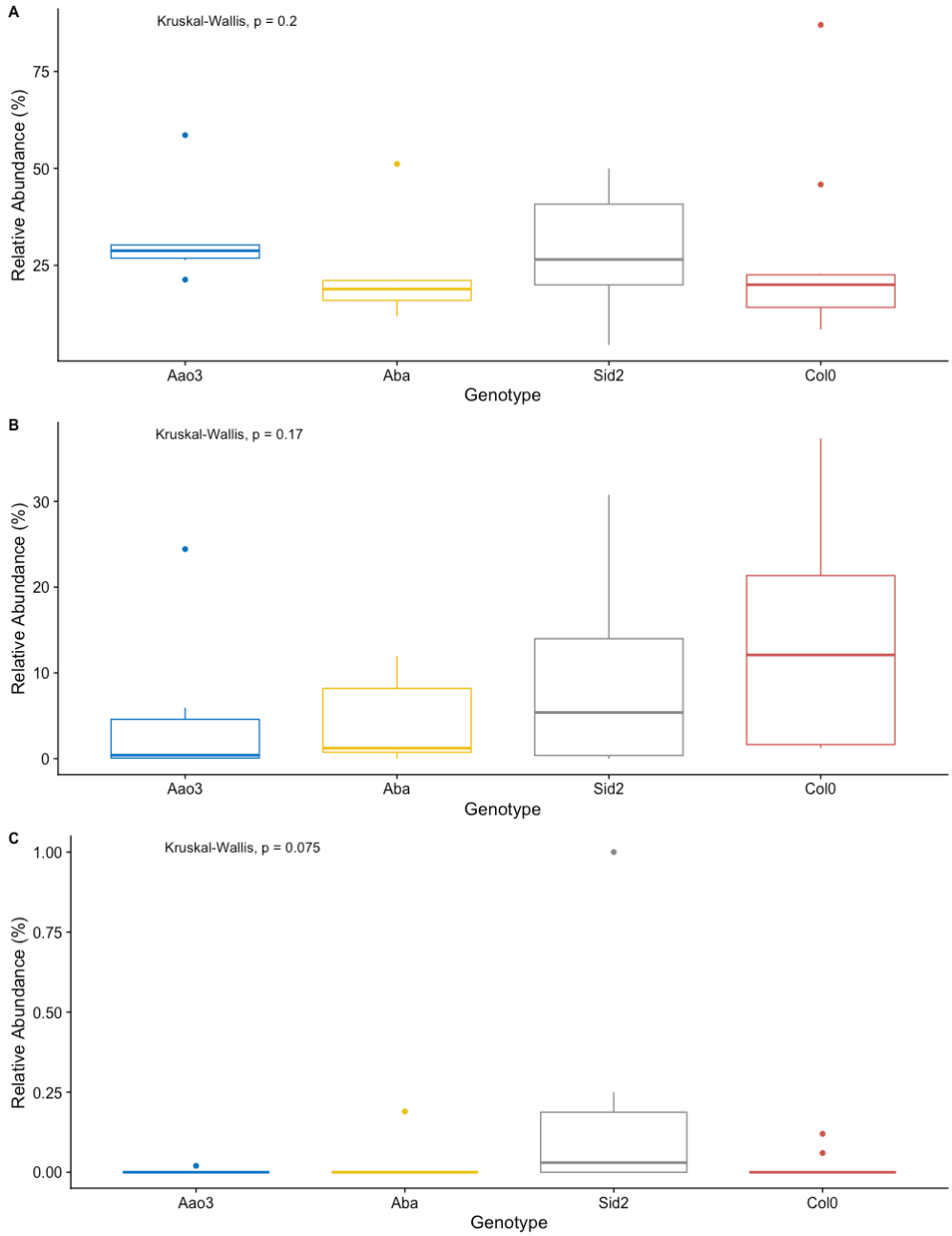

Figure 4.5 Boxplot of relative abundances of functionally significant FunGuild guilds between genotypes. *Data is representative of taxa assigned with one guild of 'highly probable' and 'probable' confidence. Guilds included are, plant pathogens (**A**), endophytes (**B**) and mycorrhizae (**C**). Statistical analysis comprised of Kruskal-Wallis non-parametric test with corresponding test statistic displayed on upper left of graph. Whiskers are representative of inter quartile range with dots representative of outliers based on interquartile range. Sample number for each genotype; Aao3 = 7, Aba = 6, Sid2 = 10, Col0 = 10.*

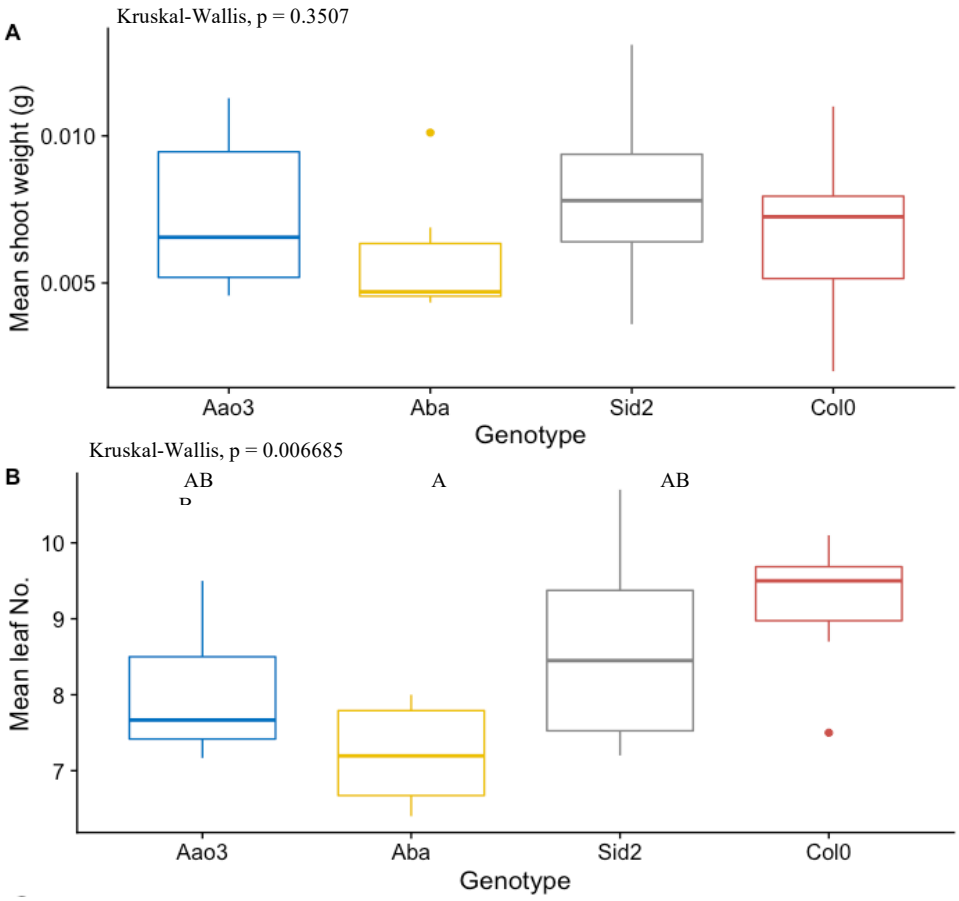

Figure 4.6 Boxplot of genotype dry shoot weight (A) and leaf number (B). *Statistical testing carried out using the Kruskal-Wallis non-parametric analysis for multiple groups with test p-value listed in top left corner of graph. Dunn's Test post-hoc analysis with Bonferroni multiple comparison correction was employed to identify significantly different comparisons. Letters are indicative of significantly different ($p < 0.05$) groups (different letters = significantly different groups). Whiskers are representative of inter quartile range with dots representative of outliers based on interquartile range. Sample number for each genotype; Aao3 = 7, Aba = 6, Sid2 = 10, Col0 = 10.*

4.4.2 Phase II: Legacy of wild-type Col0 and SA deficient Sid2 conditioned soils upon subsequent plant root mycobiomes and biomass

Col0 and Sid2 conditioned soils from the Phase I experiment were retained for use in the Phase II soil microbiome legacy experiment. In the Phase II experiment Col0 and Sid2 conditioned soils were used to a create microbial inoculant for the growth media used to grow new Col0 and Sid2 *A. thaliana* plants. To achieve this, *A. thaliana* seeds were sown into growth media treated with either the Col0 or Sid2 soil inoculant i.e., Col0 inoculant – Col0 seed, Col0 inoculant – Sid2 seed, Sid2 inoculant – Col0 seed and Sid2 inoculant – Sid2 seed. This allowed observation of whether genotype conditioned bulk soil microbiome transfer could result in significantly different root mycobiomes in wild-type Col0 and immune deficient Sid2 genotypes. It was hypothesised that wild-type Col0 soil could provide a beneficial microbiome for immune deficient Sid2 to select from and Sid2 soil a deleterious microbiome for Col0 to select from. In addition, transfer to the same genotype as the conditioning genotype could cause enrichment of deleterious or beneficial soil mycobiomes. However, in this experiment only wild-type Col0 seeds successfully germinated, only allowing comparison of Col0 soil and Sid2 Soil treatment on wild-type Col0 root mycobiomes.

4.4.2.1 Col0 and Sid2 *A. thaliana* genotypes did not condition beneficial or deleterious bulk soil mycobiomes

Bulk soil which was conditioned with wild-type Col0 or SA deficient Sid2 *A. thaliana* during the Phase I experiment (section 4.4.1) was sampled before utilisation in soil microbiome inoculants. This allowed observation of, whether bulk soil microbiomes were significantly conditioned by the growth of *A. thaliana* genotypes and whether these genotypes conditioned significantly different bulk soil microbiomes.

ASV level community diversity analysis identified that Col0 and Sid2 conditioned bulk soils were significantly different to the original field soil (Wellesbourne). However, no significant differences between Col0 and Sid2 conditioned soils were identified. NMDS ordination of Bray-Curtis $\beta-$ dissimilarity matrices showed clear clustering of Col0 and Sid2 conditioned bulk soil

away from Wellesbourne soil, with PERMANOVA analysis identifying significant ($p < 0.05$) differences in community matrix centroids (**Table 4.3**). Subsequently, ANOSIM analysis enabled pairwise comparison of soils and identified significant dissimilarity between Wellesbourne and *A. thaliana* genotype conditioned soils (**Figure 4.7A & Table 4.4**). In addition, no significant differences in community alpha diversity were identified between genotype conditioned soils and between genotype conditioned soils and Wellesbourne soil (**Figure 4.7B**).

Table 4.2 PERMANOVA analysis output for comparison of Wellesbourne and A. thaliana genotype conditioned soils β-dissimilarity matrix centroids.

	df	sum of squares	mean squares	f. model	r^2	p-value
genotype	2	0.11640	0.058202	2.2317	0.28865	0.001 (**)
residuals	11	0.28687	0.026080		0.88591	
total	13	0.40328			1.00000	

Table 4.3 Pairwise ANOSIM analysis comparison results of Wellesbourne and A. thaliana genotype conditioned soils.

comparison	r^2 value	p-value
Col0 Soil vs Sid2 Soil	0	0.473
Col0 Soil vs Wellesbourne Soil	0.516	0.010
Sid2 Soil vs Wellesbourne Soil	0.66	0.009

Community assembly analysis at lower taxonomic resolution (Class level) also reflected ASV level analyses with no significant differences identified between Col0 and Sid2 soils but significant differences between these soils and Wellesbourne soil. Comparison of taxa relative abundances showed significant differences at the Class level between genotype conditioned soils and Wellesbourne soil (**Figure 4.8**). Dothideomycetes were significantly less abundant in Sid2 soil compared to Wellesbourne (**Figure 4.8B**). Eurotiomycetes were significantly enriched in Col0 and

Sid2 soil compared to Wellesbourne (**Figure 4.8B**). Agaricomycetes were significantly enriched in Col0 soil compared to Wellesbourne (**Figure 4.8B**), and the Pezizomycetes were significantly enriched in Sid2 and Col0 soil compared to Wellesbourne (**Figure 4.8B**).

Analysis of the assembly of functionally significant fungal guilds also revealed no significant differences between Col0 and Sid2 conditioned soils. No significant differences in the relative abundances of 'highly probable' and 'probable' plant pathogens, fungal endophytes and mycorrhizas were observed (**Figure 4.9**). This is in line with Sid2 and Col0 fungal root microbiomes which also showed no significant differences in the relative abundances of functionally significant guilds (section 4.4.1.2). In summation, the wild-type Col0 and Sid2 SA deficient *A. thaliana* genotypes did not recruit or condition significantly different root and bulk soil mycobiomes.

4.4.2.2 Creation of soil microbiome inoculants introduced significant differences in mycobiomes between inoculants and original soil.

Introduction of Col0 and Sid2 conditioned bulk soil to a sterile sand clay mixture for creation of an inoculum for microbiome transfer resulted in significant differences ($p < 0.05$) between inoculate mycobiomes, in terms of community $\beta - dissimilarity$ (PERMANOVA) (Table 4.5) and NMDS clustering (Figure 4.10A). However, ANOSIM analysis (Table 4.6) and $\alpha - diversity$ (Fisher's α) (Figure 4.10B) did not identify any significant dissimilarity or differences in α diversity between inoculate mycobiome assembly.

Table 4.4 PERMANOVA analysis output for comparison of Col0 and Sid2 soils and soil inoculants β-dissimilarity matrix centroids.

	df	sum of squares	mean squares	f. model	r^2	p-value
genotype	3	0.62881	0.209605	2.2005	0.32044	0.031 (*)
residuals	14	1.33356	0.095254		0.67956	
total	17	1.96238			1.00000	

Table 4.5 Pairwise ANOSIM analysis comparison results of Col0 and Sid2 soils and soil inoculants.

comparison	r^2 value	p-value
col0 soil vs sid2 soil	0	0.473
col0 mix vs sid2 mix	0.156	0.131
col0 soil vs col0 mix	0.088	0.220
sid2 soil vs sid2 mix	0.175	0.109

Further investigation revealed that there were significant differences in the relative abundances of four fungal genera between soils and transfer inoculants driving the significant differences in community centroids identified by PERMANOVA analysis (**Figure 4.11**). The Arachnomyces genus was found to be significantly ($p < 0.05$) enriched in the Col0 inoculant (mean RAB = 1.052 %) compared to Col0 soil (mean RAB = 0.006 %) (**Figure 4.11B**). The Trichoderma genus was found to be significantly enriched in the Sid2 inoculant (mean RAB = 31.584 %) compared to Sid2 soil (mean RAB = 0.665 %) (**Figure 4.11B**). The Metarhizium genus was found to be significantly less abundant in the Sid2 inoculant (mean RAB = 0.718 %) compared to Sid2 soil (mean RAB = 2.148 %). And finally, the Saitozyma genus was found to be significantly less abundant in the Sid2 inoculant (mean RAB = 1.456 %) compared to Col0 soil (mean RAB = 3.432 %) and the Col0 inoculant (mean RAB = 3.435 %).

In addition to the significant differences in genus relative abundances, a significant difference was identified between the relative abundance of probable and highly probable plant pathogen taxa (as identified by FunGuild analysis) between Col0 Soil (mean RAB = 25.35 %) and the Sid2 soil inoculant (mean RAB = 15.376 %) (**Figure 4.12 B**). Together these results indicated that the process of inoculum production can introduce variation to mycobiome communities which are not representative of the original soil mycobiome.

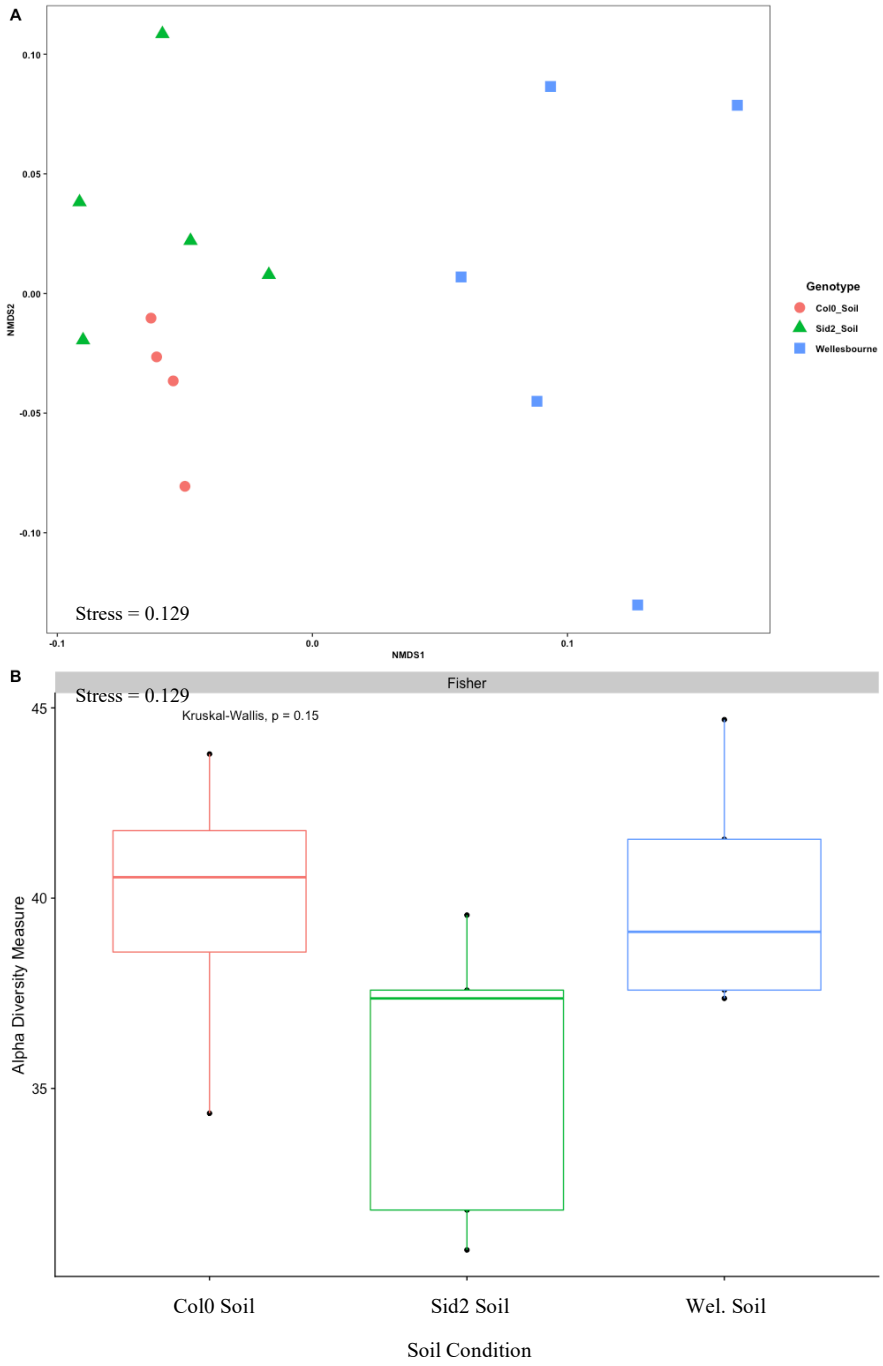

Figure 4.7 Community diversity metrics of Col0, Sid2 and Wellesbourne soils*. (A) NDMS ordination of inoculate community $\beta-$ dissimilarity matrices based on Bray Curtis dissimilarity (Stress indicated on bottom left of graph). Statistical testing carried out using ADONIS analysis of dissimilarity matrix centroids with 999 permutations (R^2 and p-value indicated on bottom left of graph). (**B**) Alpha diversity analysis using Fisher's α diversity analysis. Statistical testing carried out using the Kruskal-Wallis non-parametric analysis for multiple groups with test p-value listed in top left corner of graph. Sample number for each inoculate; Col0 Soil = 4, Sid2 Soil = 5, Wellesbourne Soil = 5.*

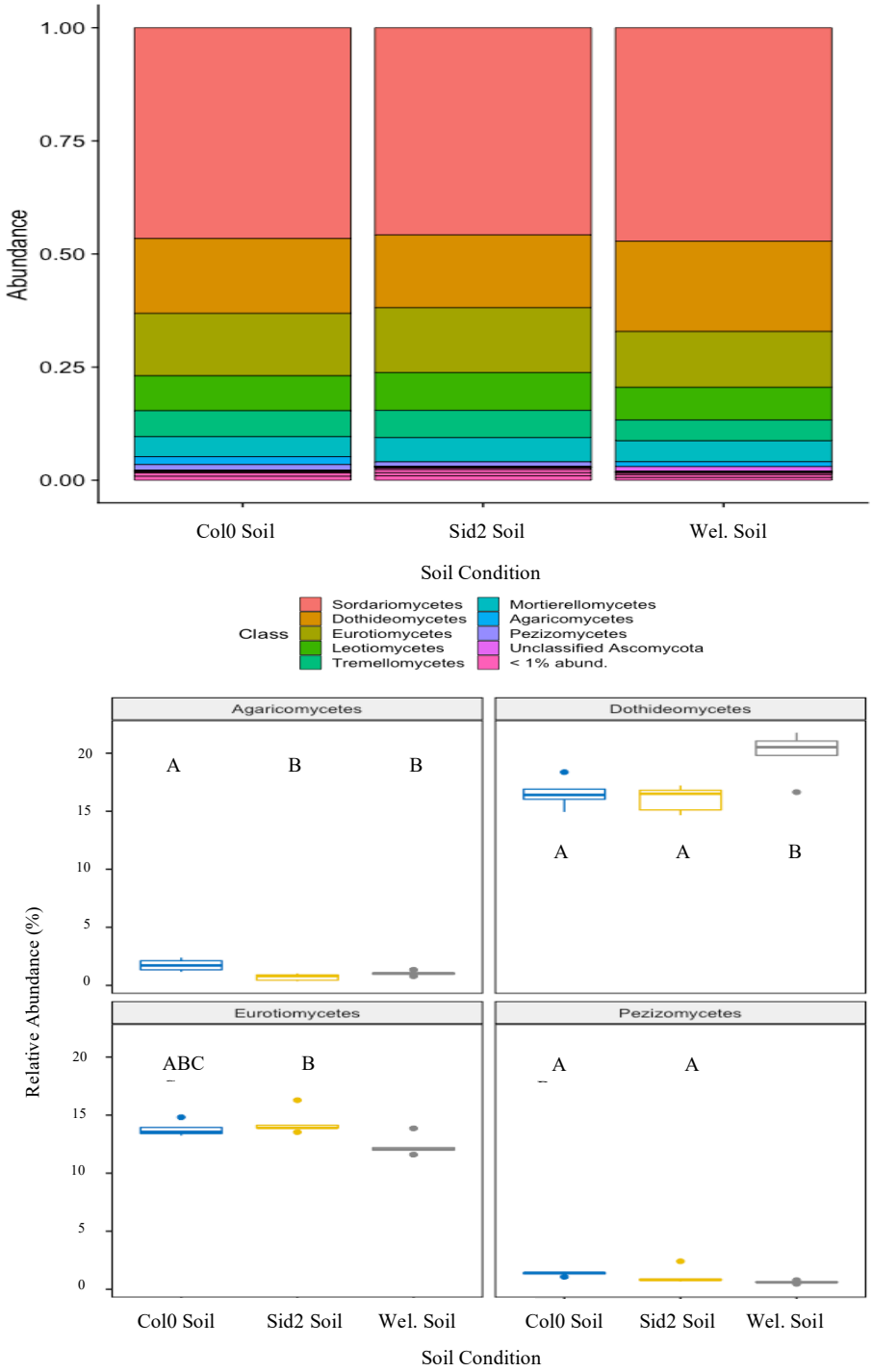

Figure 4.8 Relative abundances of soil mycobiomes grouped at the class level. *(A) Stacked bar-plot of relative abundance of soils grouped at Class taxonomic level.* ***(B)*** *Boxplots of relative abundance of significantly different ($p < 0.05$) taxa between soils. Statistical testing carried out using the Kruskal-Wallis non-parametric analysis for multiple groups with test p-value listed in top left corner of graph. Letters are indicative of significantly different ($p < 0.05$) groups (different letters = significantly different groups). Boxplot whiskers are representative of inter quartile range with dots representative of outliers based on interquartile range. Sample number for each inoculate; Col0 Soil = 4, Sid2 Soil = 5, Wellesbourne Soil = 5.*

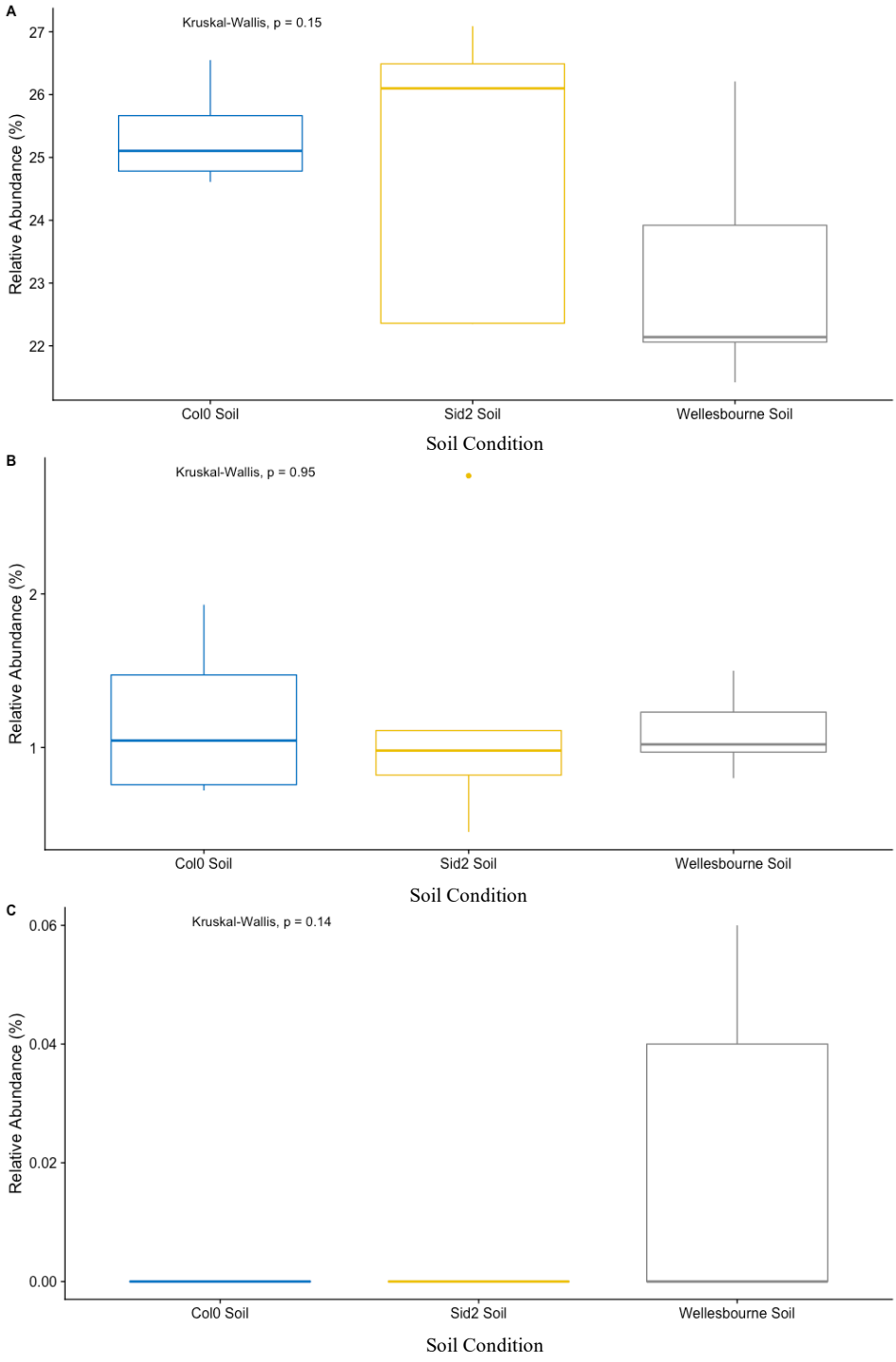

Figure 4.9 Boxplots of relative abundance of functionally significant FunGuild guilds between soils. *Data is representative of taxa assigned with one guild of 'highly probable' and 'probable' confidence. Guilds included are, plant pathogens (**A**), endophytes (**B**) and mycorrhizae (**C**). Statistical analysis comprised of Kruskal-Wallis non-parametric test with corresponding test statistic displayed on upper left of graph. Boxplot whiskers are representative of inter quartile range with dots representative of outliers based on interquartile range. Sample number for each genotype; Col0 Soil = 4, Sid2 Soil = 5, Wellesbourne Soil = 5*

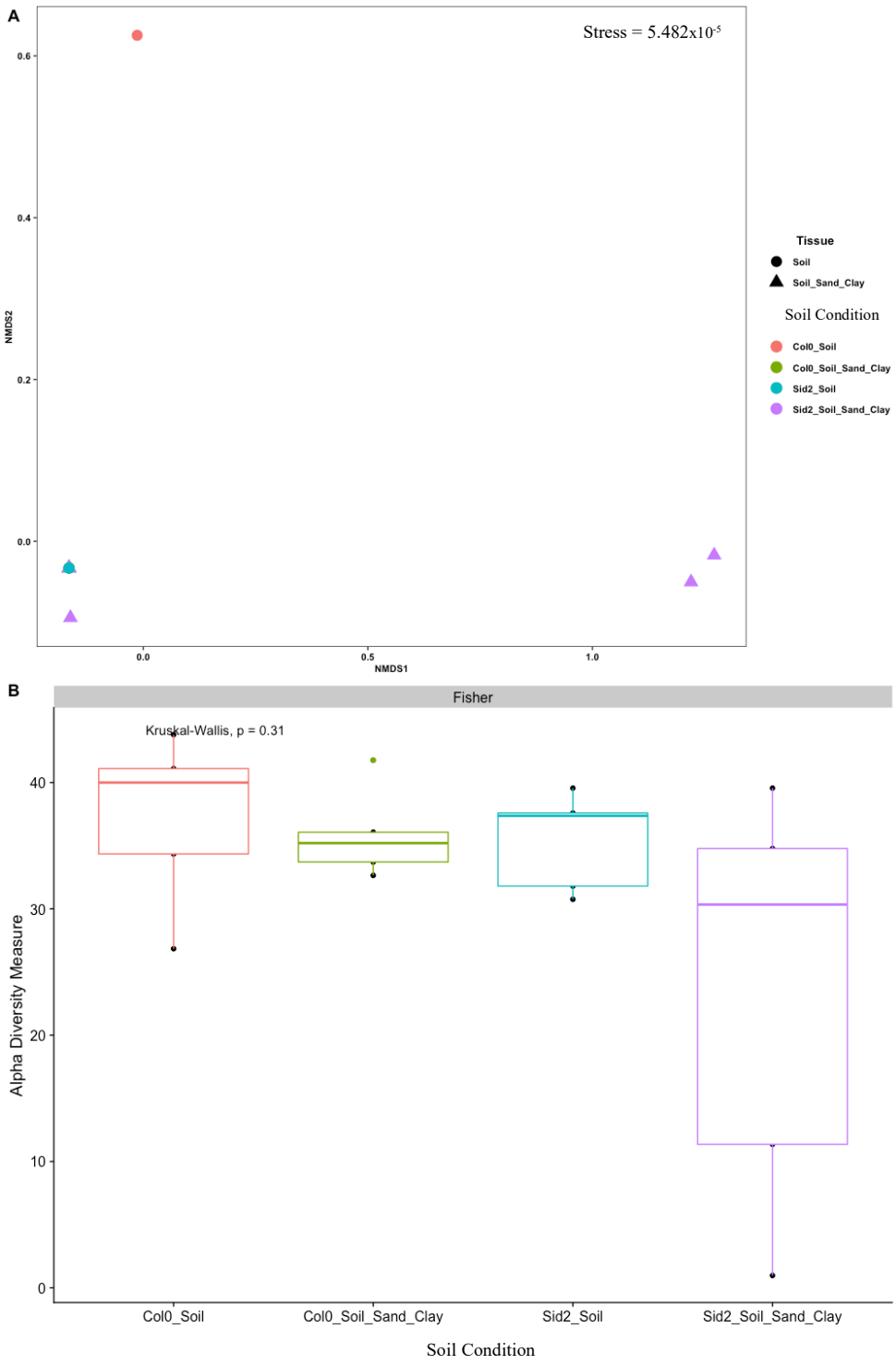

Figure 4.10 Community diversity metrics of Col0 and Sid2 inoculate. *(**A**) NDMS ordination of inoculate community $\beta - dissimilarity$ matrices based on Bray Curtis dissimilarity (Stress indicates on top right of graph). Statistical testing carried out using ADONIS analysis of dissimilarity matrix centroids with 999 permutations (R^2 and p-value indicated on top right of graph). (**B**) Alpha diversity analysis using $Fisher's\ \alpha$ diversity analysis. Statistical testing carried out using the Kruskal-Wallis non-parametric analysis for multiple groups with test p-value listed in top left corner of graph. Sample number for each inoculate; Col0 Soil = 4, Col0 Soil Sand Clay = 5, Sid2 Soil = 5, Sid2 Soil Sand Clay = 5.*

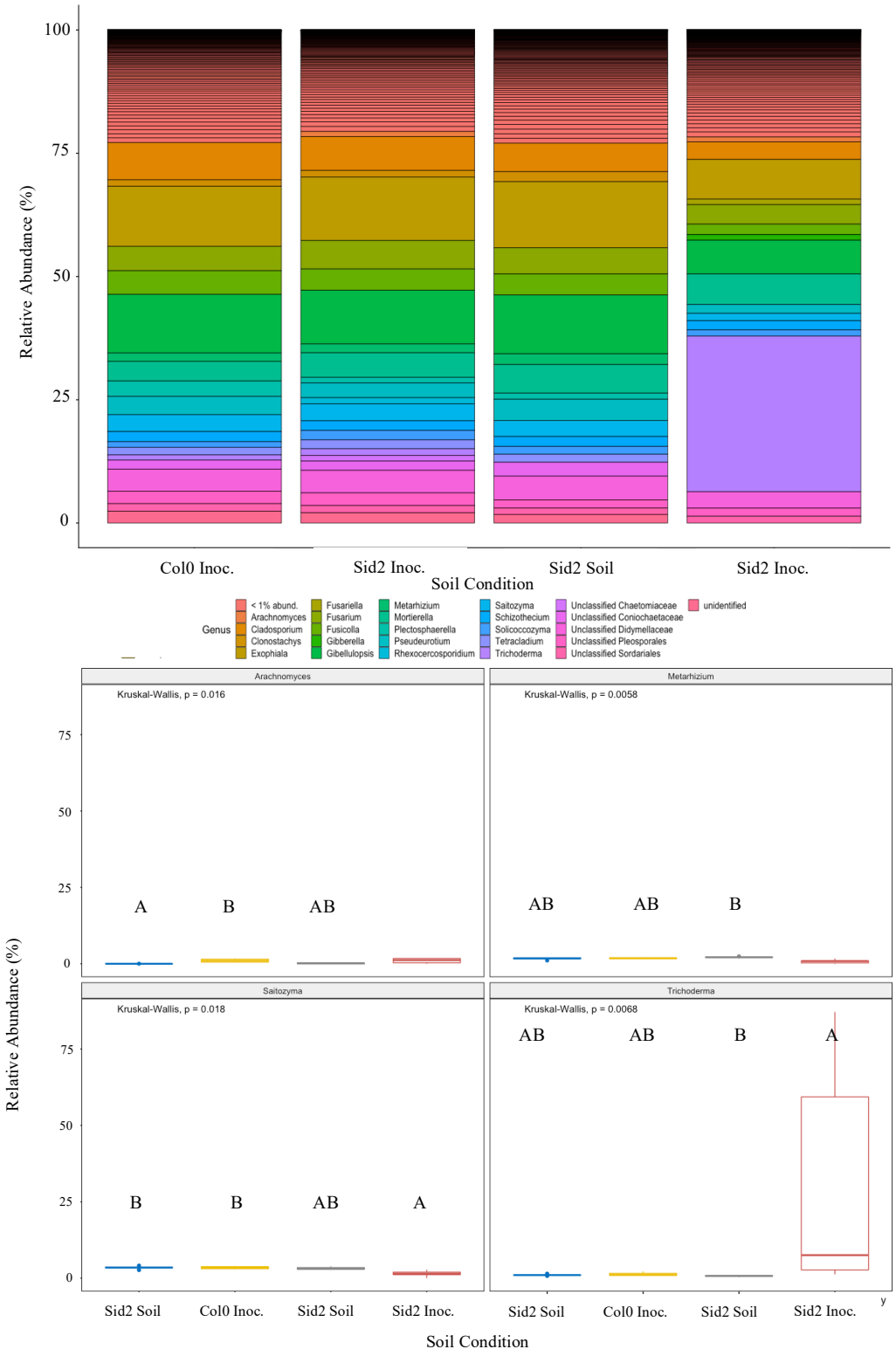

Figure 4.11 Relative abundances of soil and inoculate mycobiomes grouped at the genus level. *(**A**) Stacked bar-plot of relative abundance of soils grouped at Class taxonomic level. (**B**) Boxplots of relative abundance of significantly different ($p < 0.05$) taxa between soils. Statistical testing carried out using the Kruskal-Wallis non-parametric analysis for multiple groups with test p-value listed in top left corner of graph. Letters are indicative of significantly different ($p < 0.05$) groups (different letters = significantly different groups). Boxplot whiskers are representative of inter quartile range with dots representative of outliers based on interquartile range. Sample number for each inoculate; Col0 Soil = 4, Sid2 Soil = 5, Col0 Soil Sand Clay = 5, Sid2 Soil Sand Clay = 5*

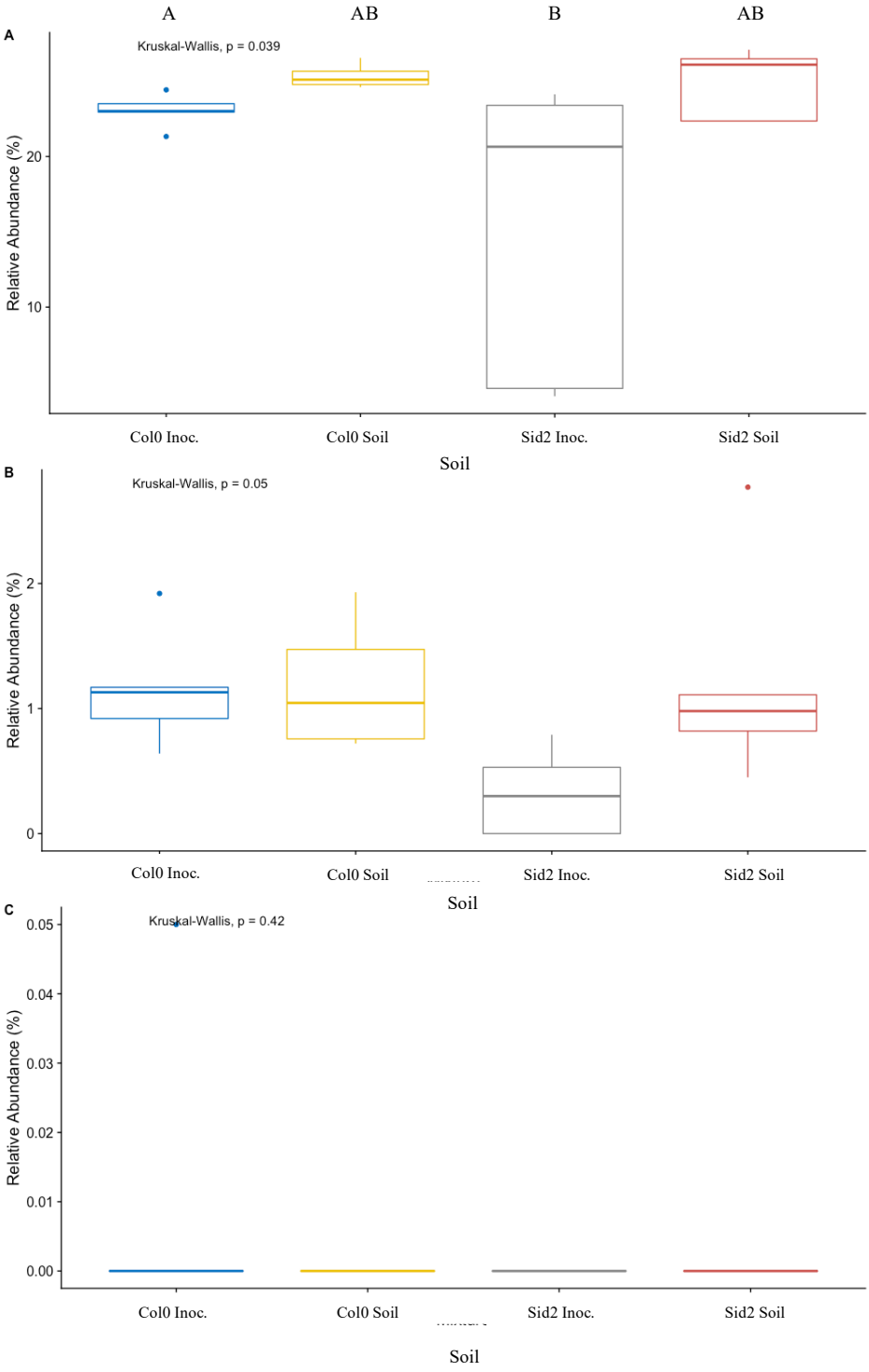

Figure 4.12 Relative abundance of functionally significant FunGuild guilds between soils and inoculates. *Data is representative of taxa assigned with one guild of 'highly probable' and 'probable' confidence. Guilds included are, plant pathogens (**A**), endophytes (**B**) and mycorrhizae (**C**). Statistical analysis comprised of Kruskal-Wallis non-parametric test with corresponding test statistic displayed on upper left of graph. Statistical testing carried out using the Kruskal-Wallis non-parametric analysis for multiple groups with test p-value listed in top left corner of graph. Letters are indicative of significantly different ($p < 0.05$) groups (different letters = significantly different groups). Sample number for each genotype; Col0 Soil = 4, Col0 Soil Sand Clay = 5, Sid2 Soil = 5, Sid2 Soil Sand Clay = 5.*

4.4.2.3 Transfer of Col0 or Sid2 conditioned soils did not significantly alter Col0 root mycobiome assembly or plant biomass parameters

Despite the significant differences between soil inoculant mycobiomes observed in the previous section (section 4.4.2.1), there were no significant differences in the fungal root microbiomes in plants grown in Col0 conditioned and Sid2 conditioned soil inoculant media.

No significant differences in community $\beta-$ diversity metrics were observed with NMDS and PERMANOVA analysis showing no significant differences in $\beta-$ dissimilarity matrix centroids between Col0 soil and Sid2 soil treated Col0 plants (**Figure 4.13A & Table 4.7**). In addition, no significant differences in Fisher's α diversity was observed between Col0 soil treated Col0 and Sid2 soil treated Col0 fungal root mycobiomes (**Figure 4.13B**).

Regarding community composition, no significant differences in taxa relative abundances were observed between Col0 soil treated Col0 and Sid2 soil treated Col0 fungal root mycobiomes (**Figure 4.14**). There were also no significant differences identified between Col0 treatments in the relative abundances of 'highly probable' and 'probable' plant pathogens, fungal endophytes, and mycorrhizal fungi (**Figure 4.15**). In addition, soil treatment had no effect upon plant biomass parameters including dry shoot weight and average leaf number (**Figure 4.16**). There were also no significant correlations between ASVs and any of these biomass parameters. Together, these results indicate that despite significant differences between Col0 and Sid2 conditioned soil inoculants, these inoculants did not have a significant effect upon the formation of Col0 root mycobiomes.

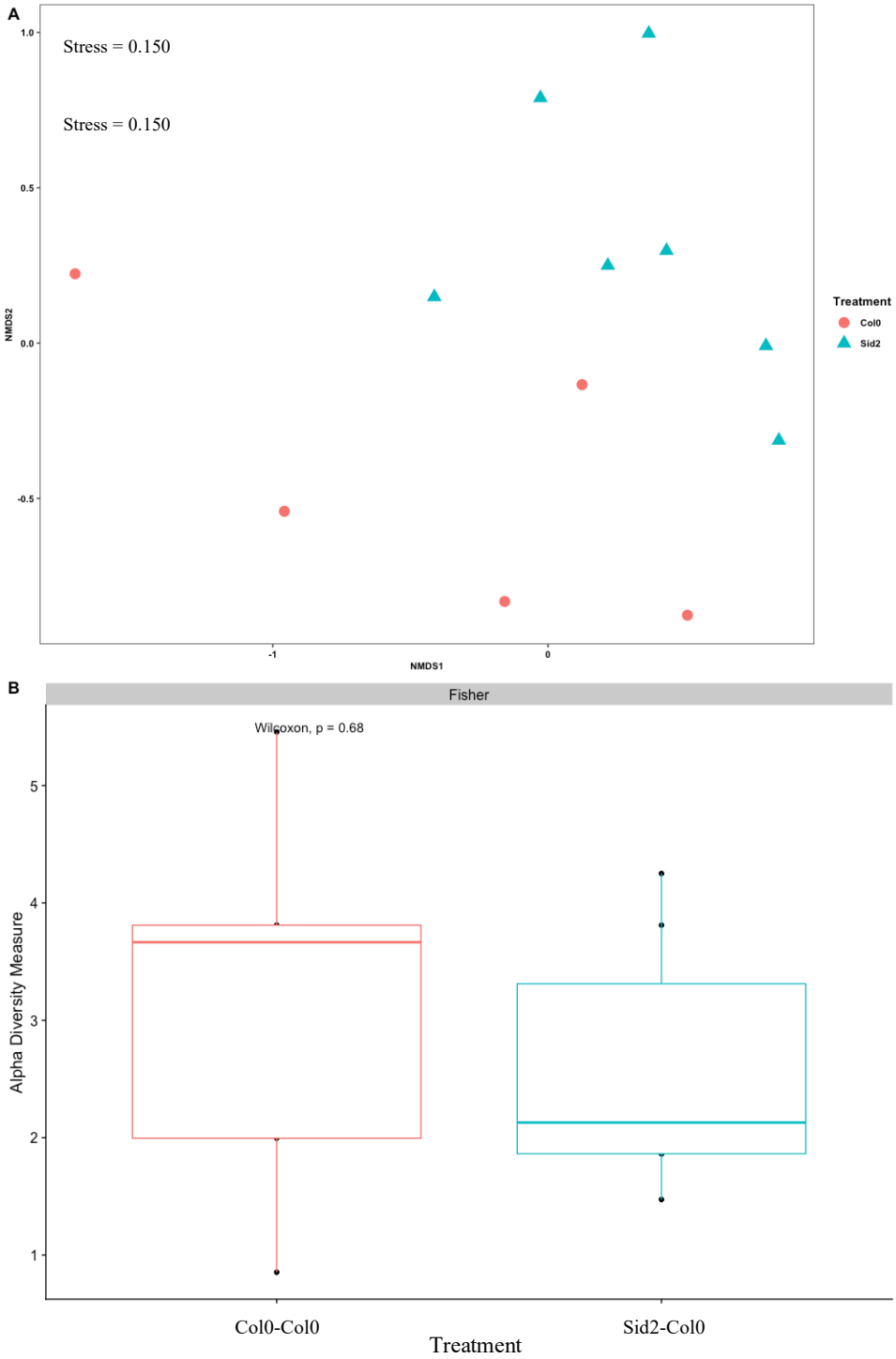

Figure 4.13 Community diversity metrics of Col0 and Sid2 soil treated Col0 fungal root microbiomes *(**A**) NDMS ordination of inoculate community $\beta-$dissimilarity matrices based on Bray Curtis dissimilarity (Stress indicates on top right of graph). Statistical testing carried out using ADONIS analysis of dissimilarity matrix centroids with 999 permutations (R^2 and p-value indicated on top right of graph). (**B**) Alpha diversity analysis using Fisher's α diversity analysis. Statistical testing carried out using the Wilcoxon Rank sum non-parametric analysis with test p-value listed in top left corner of graph. COL0 = Col0 Soil treated Col0, SID2 = Sid2 Soil treated Col0) Sample number for each inoculate; Col0-Col0= 5, Sid2-Col0 = 7.*

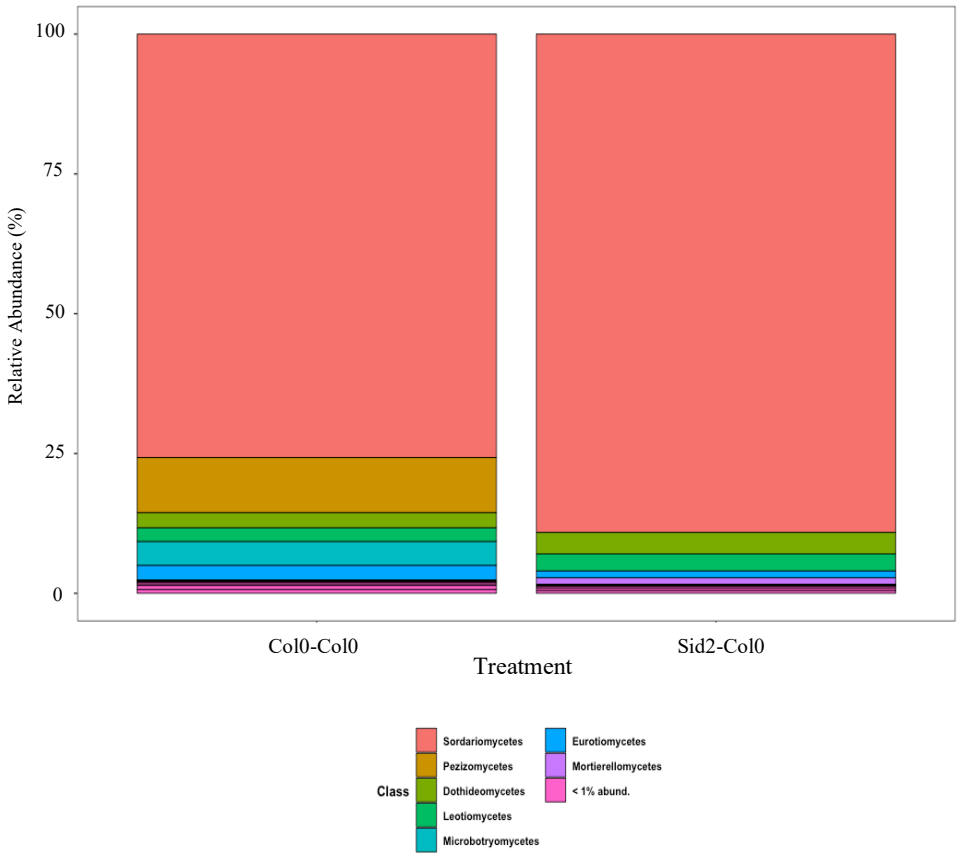

Figure 4.14 Stacked Bar plot of community relative abundances. *(A) Relative abundance of treated Col0 root microbiomes grouped at Order taxonomic level and grouped by treatment Sample number for each inoculate; Col0-Col0= 5, Sid2-Col0 = 7.*

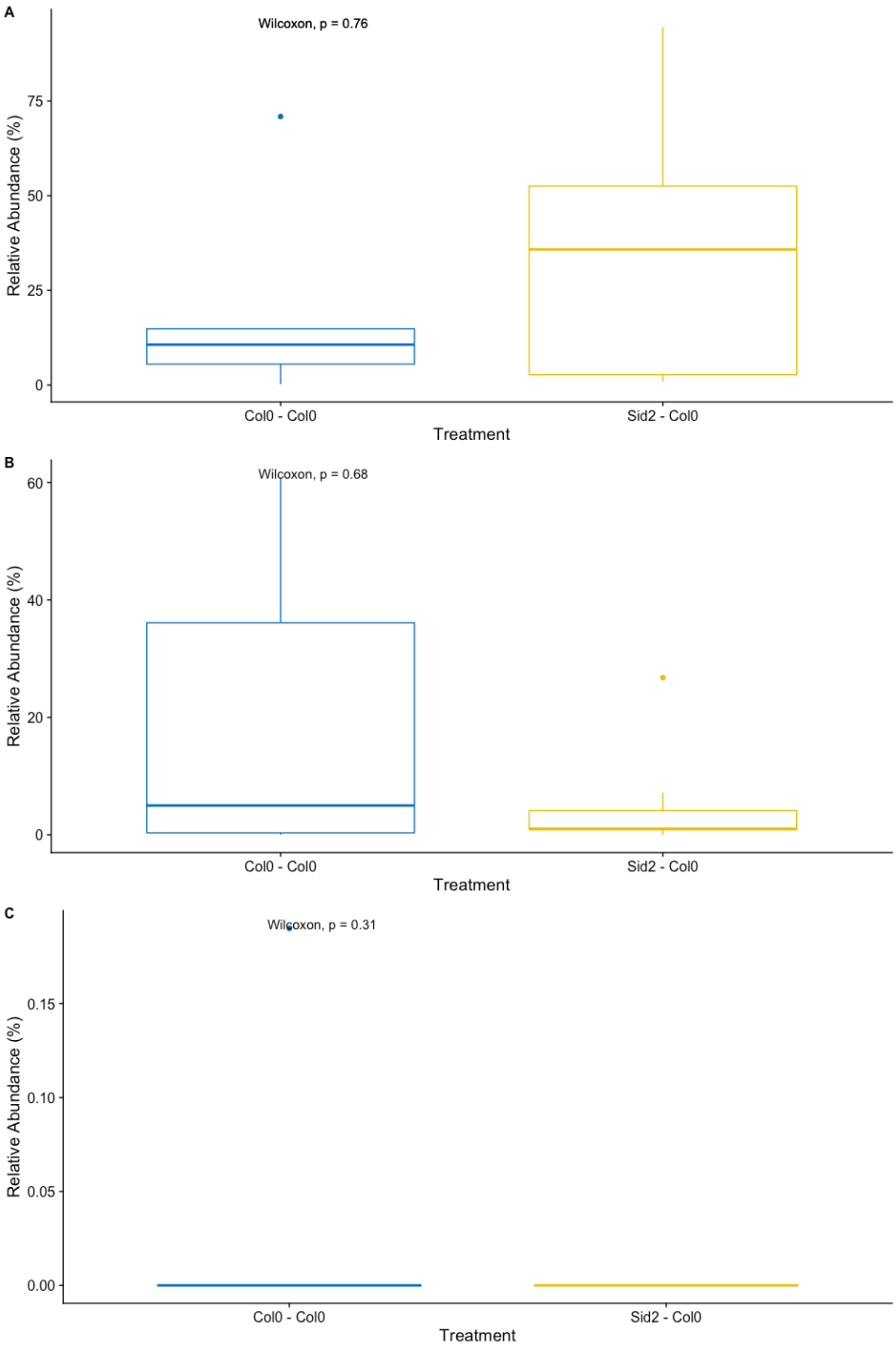

Figure 4.15 Relative abundance of functionally significant FunGuild guilds between treated Col0 root microbiomes. *Data is representative of taxa assigned with one guild of 'highly probable' and 'probable' confidence. Guilds included are, plant pathogens (**A**), endophytes (**B**) and mycorrhizae (**C**). Statistical analysis comprised of Wilcoxon Rank Sum non-parametric test with corresponding test statistic displayed on upper left of graph. Sample number for each inoculate; Col0-Col0= 5, Sid2-Col0 = 7.*

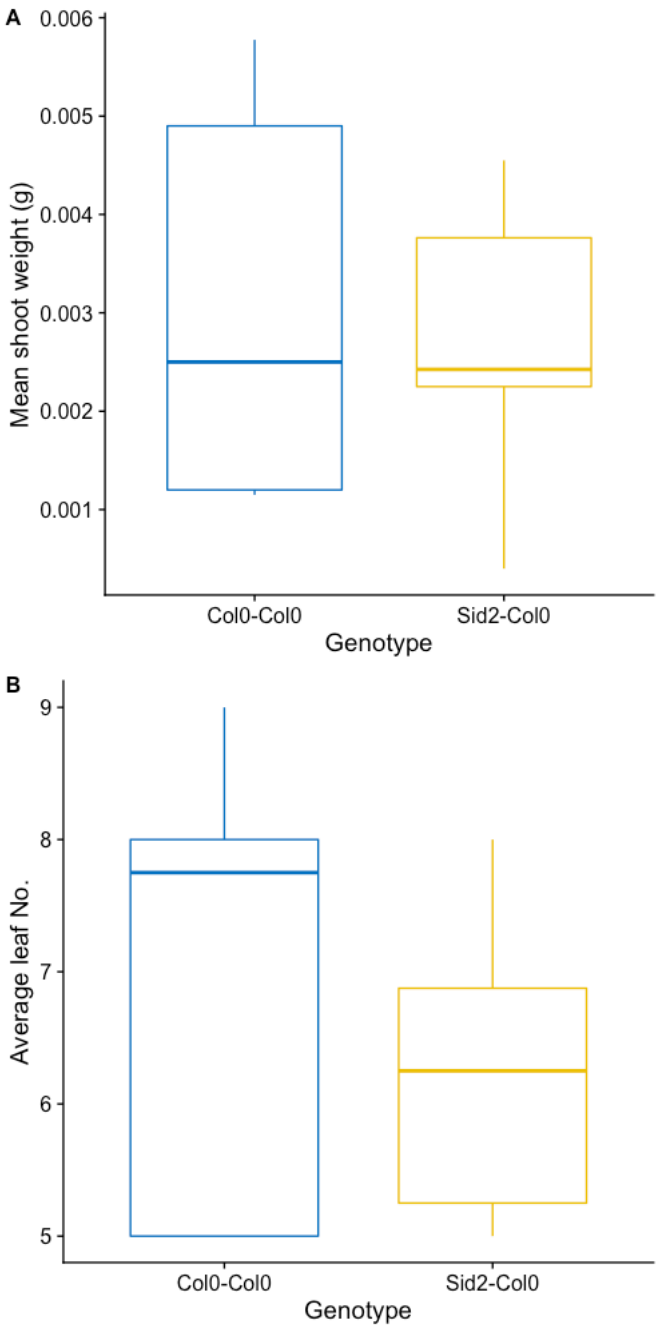

Figure 4.16 Boxplot of genotype dry shoot weight (A) and leaf number (B) in microbiome transfer treated Col0. *Statistical testing carried out using the Wilcoxon Rank Sum non-parametric analysis with test p-value listed in top left corner of graph. Sample number for each inoculate; Col0-Col0= 5, Sid2-Col0 = 7.*

4.5 Discussion

4.5.1 ABA mutant *A. thaliana* genotypes have significant enrichment of Basidiomycota and/or Sordariales

It has already been established that deficiencies in JA and SA can lead to the development of distinct bacterial root and rhizosphere soil microbiomes in *A. thaliana* and *T. aestivum* (Carvalhais et al., 2013, Carvalhais et al., 2015, Lebeis et al., 2015, Liu et al., 2017). It has also been observed that JA deficiency can lead to increased mycobiome diversity in *S. lycopersicum* (Manzotti et al., 2020). However, this research presents the first information on fungal community assembly in response to SA deficiency and is the first piece of research which examines any microbial communities' assembly in response to ABA deficiency and hypersensitivity.

It was observed in this study that genotypes deficient in or hypersensitive to ABA were associated with having significantly higher relative abundances of Basidiomycota in the root mycobiome compared to wild-type *A. thaliana*. In addition, the ABA deficient genotype was found to have significantly higher relative abundance of Sordariales compared to wild-type *A. thaliana*. Regarding the Basidiomycota, they are functionally diverse taxonomic grouping of fungi containing many plant pathogens such as rusts and smuts (Dugan, 2017), beneficial ectomycorrhizal species (Watkinson, 2008) and endophytes known to confer pathogen protection upon *A. thaliana* (Sun et al., 2014). However, since no significant differences in Basidiomycete taxa were identified at higher taxonomic resolutions, it is difficult to hypothesise what this increase in total Basidiomycota may mean for the plant host. Regarding the Sordariales, they are an Order of fungi within the Ascomycete Phylum which are saprobic soil inhabiting organisms (Eriksson et al., 2001). As discussed with the Basidiomycota taxa, it is difficult to hypothesise what this may mean for the plant host. This is especially true given that there were no significant differences in community α or β diversity indices, relative abundances of fungal functional guilds and no significant correlations between taxa and plant biomass.

Given these findings, it cannot be said that there is a formation of 'deleterious' fungal root microbiomes in SA and ABA immune mutant *A. thaliana* genotypes based on the analysis techniques used in this study. Shifts in the abundance of certain phyla were identified, however, these were not associated with significant differences in

plant biomass parameters or common pathogenic fungal taxa. However, the limitations of the analyses used in, and the scope of this study also limit a wholistic understanding of whether 'deleterious' microbiomes form in response to these plant immune mutant genotypes.

Firstly, restriction of microbiome analysis to root tissues prevented observation of whether rhizosphere soil mycobiome assembly is altered between immune mutant genotypes. Secondly, the use of functional inference based on taxonomy alone through FunGuild analysis is inherently limited by the constraints of the FunGuild database and molecular investigations of fungi in general (Nguyen et al., 2016b, Pozo et al., 2021). For example, most taxa included in the FunGuild database are assigned to the genus level, reducing the probability of correct guild assignment as species level assignments are most accurate. In addition, approximately 148,000 fungal species have been taxonomically described whereas, estimates of the total number of species are between 2.2 to 3.8 million and up to 12 million (Hawksworth and Lücking, 2017, Wu et al., 2019). Therefore, this low level of taxonomic coverage limits the ability of functional FunGuild assignment techniques. Finally, despite this study being principally concerned with filling gaps in our understanding of fungal community assembly, exclusion of bacterial and protist communities from analyses precluded the ability to construct inter-taxon co-occurrence networks. Fungi have been observed to be keystone hub taxa in such networks constructed from *A. thaliana* microbiomes, with inter-kingdom interaction in roots found to be critical to *A. thaliana* survival (Durán et al., 2018). Therefore, examination of inter-kingdom interaction in immune-phytohormone mutants can be seen as a key analysis in future investigation. Despite the limitations of this study, seminal results for fungal microbiome assembly in immune-phytohormone mutant plant genotypes are presented with study limitations providing a range of avenues for further research.

4.5.2 Soil transfer inoculant creation significantly altered mycobiome assembly, however no differences in treated root mycobiome assembly were identified

As discussed in section 4.5.2, no significant differences in root fungal community assembly were identified between SA deficient (Sid2) and wild-type

(Col0) *A. thaliana*. This was also observed between bulk soils conditioned with either Col0 or Sid2 deficient *A. thaliana* in Phase I of experimentation. However, it was observed that both Col0 and Sid2 treated soils were significantly different to the original Wellesbourne soil inoculum used at the start of Phase I. This indicated that Sid2 SA deficient genotype did not assemble a significantly different bulk soil mycobiome compared to wild-type Col0. Significant differences in community β-diversity was observed between Wellesbourne and conditioned soils with significant differences in the relative abundances of the Agaricomycetes, Dothideomycetes, Eurotiomycetes and Pezizomycetes identified. This is unsurprising given that it is known that plants can influence the formation of the surrounding bulk soil microbiome (Berendsen et al., 2012, Lareen et al., 2016). However, no differences in α-diversity or the relative abundances of functionally significant fungal guilds were identified between Col0, Sid2 and Wellesbourne soil.

Inoculants created from the Col0 and Sid2 conditioned soils were found to have no significant differences in community diversity or assembly compared to the original soils. However, significant differences were identified in the relative abundance of certain taxa. Of note, was the significant enrichment of the Trichoderma Genus in the Sid2 inoculant. Also, the Sid2 inoculant was found to have significantly lower abundance of Pathogenic taxa. This reflected variation introduced by inoculum creation. Indeed, divergence in community assembly from inoculum creation has already been observed in previous research and highlights the potential drawbacks of creating soil microbial inoculants (Howard et al., 2017). Despite this introduced variation, no significant differences were found between Col0 root mycobiomes that were treated with Col0 or Sid2 inoculant. Therefore, no microbiome legacy effects including 'rescuing' or 'compounding' effects upon subsequent plants could be observed using the techniques employed in this study.

4.5.3 Directions for future research

The research presented here provides seminal findings for fungal microbiome assemblies in SA deficient and ABS deficient and hypersensitive plants using the model plant species *A. thaliana*. However as previously discussed, there are several

limitations to the research presented here which provides challenges and opportunities for future research.

Regarding analysis of microbiome assemblies and functionality, a number or different techniques and experimental designs may be employed to navigate the limitations of the techniques used in this study. Firstly, as previously discussed, inclusion of bacteria and protist taxa in investigations could allow the identification of shifts in key inter-kingdom interactions involving fungi between immune-phytohormone genotypes. Such inter-kingdom interactions have previously been observed to be key to *A. thaliana* survival (Durán et al., 2018), and therefore represent an important avenue of research when investigating plant immune system – microbiome dynamics. Secondly, utilisation of other 'multi-omics' techniques including, shotgun metagenomics, transcriptomics and proteomics could help elucidate functional shifts in the root microbiome and plant host which are not possible to observe or infer using amplicon sequencing alone (Baldrian, 2019). However, these other multi-omics techniques are not without their own limitations and can present challenges when researching fungi due to the smaller number of full sequenced fungal genomes compared to bacteria (see section 1.2.4.3) (Baldrian, 2019, Hibbett et al., 2013, Pozo et al., 2021). Finally, alteration of experimental parameters including challenging plants with inoculates of known pathogenic taxa and/or inducing abiotic stress thorough drought or salt stress could be considered. This would allow examination of whether microbiomes and the plant host together respond differently to such stresses due to differences in immune-phytohormone expression. Such modifications may be particularly useful when examining 'rescuing' or 'compounding' effects of transferred microbiome inoculants on subsequent plants as was attempted in this study.

5 General Discussion

5.1 Research findings

The rhizosphere microbiome is of great importance to plant health and function having been described as the plants 'second genome' and an essential component in the host plants biology, ecology and evolution (Berendsen et al., 2012, Hassani et al., 2018, Simon et al., 2019). Subsequently, understanding the assembly and function of rhizosphere microbiomes can be seen as essential to the development of modern agriculture with the aim of developing microbial based agricultural tools (Busby et al., 2017, Chen et al., 2018, Toju et al., 2018, Trivedi et al., 2017, Yu et al., 2019) . However, a systematic understanding of how the microbiota assembles and functions as a whole in relation to the plant host is not fully developed. This is a key knowledge gap in the understanding of the rhizosphere microbiome and a major roadblock to the development of functional microbiome manipulation as an agricultural tool. To address this, the overall aims of this work were to develop understanding of rhizosphere microbial assembly, interactions, and function in relation to host plant genetic diversity, immune phytohormones and plant biomass. This was carried out using two members of the Brassicaceae plant family: the globally important crops species *B. napus* (OSR) and the scientifically important model organism *A. thaliana*. Together the results presented in this thesis develop our understanding of plant – microbiome assembly and functionality in the Brassicaceae and plants in general.

In Chapter two, rhizosphere community assembly, microbial interactions and impact on plant biomass and yield were assessed in relation to host plant genetic diversity. This was achieved through cultivation of four distinct OSR genotypes in a large-scale field study under standard agronomic conditions. These genotypes were selected as representatives of root biomass and plant metabolite exudate extremes in OSR. Next generation DNA amplicon sequencing was employed to assess community assembly and infer microbe-microbe interactions. This investigation developed our current understanding of plant genotype – microbiome dynamics in OSR beyond previous investigations (Bazghaleh et al., 2020, Floc'h et al., 2020, Taye et al., 2020), through assessment of: bacterial, fungal and protist microbiomes, microbe – microbe interactions and the microbiomes relationship to yield.

Comparison of microbiomes between the assessed genotypes indicated that OSR genotype did not alter microbiome composition at the community level. This is at odds with previous investigations which identified genotype influences at the community level in bacterial (Taye et al., 2020) and fungal (Bazghaleh et al., 2020) rhizosphere microbiomes of OSR. However, differentiation was observed at the OTU level between the genotypes assessed in this study. Each genotype was found to have indicator OTUs which were significantly fidelitous to each genotype's microbiome. These included taxa which are known plant pathogens such as Pythium species, Phoma species and Fusarium species (Bennett et al., 2018, Martin and Loper, 1999) and, potentially beneficial endophytes of OSR such as Fusarium species and Tetracladium species (Card et al., 2015, Hilton et al., 2021).

Co-occurrences between OTUs were also assessed using inter-taxon co-occurrence networks. These networks are constructed from significantly co-occurring and co-excluding OTUs and have previously been shown to identify functionally and ecologically significant microbe-microbe interactions in the rhizosphere (Wang et al., 2017). This analysis identified distinct network structures between genotypes despite most taxa being shared between genotype networks. Shared OTUs also shifted network roles between genotype networks with differing levels of connectivity. This suggests that despite compositional similarity, interactions between taxa and the functions they have in each genotype's microbiome may change. This is potentially significant since highly connected 'Network Hub' taxa have been considered 'keystone' taxa upon which microbiome structure and resilience is linked (Banerjee et al., 2018). Indeed, beneficial inter-taxon relationships have been found to be essential to plant health and disease resistance in the OSR relative *A. thaliana* (Durán et al., 2018).

In addition, to assessment of microbial assembly in response to host genotype, the relationship between microbiome assembly and OSR biomass and yield was investigated. No individual taxa were found to be significantly correlated with yield in any genotype, however, root bacterial community diversity and fungal rhizosphere soil community diversity were found to be a significant explanatory variable of yield or OSR biomass. Therefore, this analysis identified a relationship between OSR biomass values and microbial community diversity in certain genotypes of OSR under agriculturally relevant conditions.

In the context of current literature, these analyses indicated that genotype – microbiome dynamics are complex and nuanced with genotypic influences at the community assembly level potentially overwhelmed by other factors known to influence rhizosphere microbiome assembly in a real-world field setting including; edaphic factors (Schlatter et al., 2019), meteorological conditions (Brown et al., 2019, He et al., 2017), plant developmental stage (Rybakova et al., 2017) and agronomic practices (Berry and Spink, 2006, Hilton et al., 2013, Lay et al., 2018a, Rathore et al., 2017). However, the findings presented in this study show the potential for genotype-based influences beyond taxonomic assembly at the community level with distinct co-occurrence networks and indicator OTUs identified for each genotype. In addition, this study presents analysis of shifting roles of network OTUs for the first time, showing that compositionally similar networks can have shifts in OTU network functionality.

The influence of the plant host upon shaping rhizosphere microbiome assembly was also investigated in Chapter four. This comprised of an investigation into the role of immune phytohormones in root mycobiome assembly in the OSR relative *A. thaliana*. In this chapter, wild-type Col0 and three immune phytohormone mutant *A. thaliana* genotypes with either, hypersensitivity to ABA, deficiency in ABA production and deficiency in SA production were analysed. This research presented the first data on rhizosphere microbiome assembly in response to ABA and the first data on fungal microbiome assembly in response to SA deficiency. This chapter also presented the first research into the legacy effects of immune phytohormone deficiency upon subsequent *A. thaliana* root mycobiomes. This was achieved through two distinct experimental phases with phase I examining community assembly in each genotype. Whereas phase II concerned examination of the microbial legacy of the SA deficient and wild-type genotypes through creation of soil inoculums for transplantation to SA deficient and wild-type *A. thaliana*.

From phase I data, the root mycobiomes of the immune mutant genotypes were not found to have significantly different fungal communities compared to wild-type based on community diversity indices or relative abundances of functionally significant taxa. However, both ABA mutant genotypes were found to have significant enrichments of the Basidiomycota compared to SA mutant and wild-type genotypes. In addition, the ABA deficient genotype was found to have significantly higher

relative abundance of Sordariales compared to wild-type and SA deficient *A. thaliana*. However, no significant differences in any parameter were identified between wild-type and the SA deficient genotype.

This was reflected in the phase II experiment with no significant differences in SA deficient and wild-type conditioned soils being identified. However, significant differences were observed between the SA deficient genotypes soil inoculum and the original soil. This indicated that the creation of a microbial inoculant for transplantation significantly altered the resident fungal community. Also, the soil inoculum created from SA deficient genotype conditioned soil was found to have significantly lower abundance of Pathogenic taxa, reflecting that variation introduced by inoculum creation influenced the assembly of functionally significant taxa. Indeed, this has already been observed in previous research and highlights the potential drawbacks of creating soil microbial inoculants (Howard et al., 2017). Subsequently, no significant differences in community composition were identified in wild-type *A. thaliana* treated with SA deficient or wild-type conditioned soils.

Together, the data presented in Chapter Four indicated that deficiency in SA did not result in a significantly different mycobiome assembly compared to wild-type *A. thaliana* i.e., SA deficiency did not result in the formation of a 'deleterious' root mycobiome. Subsequently, no significant impact on SA conditioned soils compared to wild-type was observed, indicating that there was no legacy of a 'deleterious' soil microbiome. This is in comparison to previous research which has shown that SA deficiency resulted in significantly different bacterial root microbiomes (Lebeis et al., 2015). This indicates that SA may not have as important a role in root mycobiome formation or SA deficiency is masked by a compounding factor with stronger influence on mycobiome assembly. Regarding ABA, it was shown that deficiency in or hypersensitivity to ABA resulted in a significant enrichment of Basidiomycota, indicating a potential role of ABA expression and regulation in forming the root mycobiome.

Together, the data presented in Chapters Two and Four concerned investigations of rhizosphere microbiome assembly in response to genetic variation in the plant host i.e., to OSR cultivars with distinct root architecture and exudate phenotypes and, to *A. thaliana* genotypes with mutated immune phytohormone pathways. This data mainly addressed, microbial assembly and community

composition, however, functional analysis based on the amplicon sequence data was also carried out using correlation and FunGuild analysis. Microbial functional analysis based on these methods can give insight into potential ecological function of OTUs and thus microbial taxa. However, it should be noted that there are limitations to functional inference based on amplicon sequencing data. For example, in functional assignment of fungal taxa, the seminal paper for the FunGuild database identifies three key limitations to FunGuild analysis (Nguyen et al., 2016b). Firstly, databases are restricted in their guild descriptions, secondly, the majority of database taxa are limited to the Genus level and, only a small portion of the predicted diversity of fungal species have been taxonomically described (Hawksworth and Lücking, 2017, Wu et al., 2019). Thirdly, the lack of functional ecological description of fungal taxa in general limits the use of functional assignment based on sequence data. As such, studies which integrate community sequencing data, isolate cultivation and experimentation are necessary to, develop our understanding of fungal ecology in the genomics era and improve sequence based functional ecological assignment techniques (Nguyen et al., 2016b, Peay, 2014).

This methodology was implemented in Chapter three, in which the functionality of OSR isolated fungal taxa were assessed. In this chapter, a taxonomically diverse fungal isolate collection was obtained from OSR rhizosphere soil and root tissues. These isolates were referenced against the UK wide OSR fungal microbiome dataset published in (Hilton et al., 2021) in order to select ecologically and functionally relevant fungal isolates for investigation in plant – microbe co-inoculation studies. In total, 74 fungal isolates were obtained and were subsequently found to be representative of the UK OSR rhizosphere microbiome. These isolates included taxa which were found to be widely distributed, abundant and significantly correlated to OSR yield in UK OSR mycobiomes (Hilton et al., 2021).

Co-inoculation studies using these taxa, identified 6 isolates which had a significant deleterious impact on OSR seedling biomass after 4 weeks. These isolates included a highly abundant and distributed taxon in OSR rhizosphere microbiomes (Uncultured Mortierella), known OSR and plant pathogens (*L. biglobosa brassicae*, *Phomopsis sp.* & *Penicillum sp.*) and an 'undefined saprotroph' (*G. pannorum*). The identification of deleterious effects from co-inoculation with *G. pannorum* is particularly interesting due to its widespread distribution in global soils and the lack

of functional analysis in plants (Arenz et al., 2006, Domsch et al., 1980, Hayes, 2012). Interestingly, another OTU related to the Uncultured Mortierella isolate and isolates significantly correlated with OSR yields (*T. maxilleforme* & Uncultured Microdochium) had no significant impact on biomass, indicating species specific effects on biomass and alternative mechanisms for impacting yield other than impacts on OSR biomass during the early growth stages.

In conclusion this chapter presents the most comprehensive functional analysis of OSR rhizosphere mycobiomes through the isolation and investigation of a diverse fungal culture collection. This culture collection was found to be representative of the UKs OSR rhizosphere mycobiome through referencing isolate sequences against Hilton et al., 2021's investigation of OSR rhizosphere microbiome assembly across the UK. Hilton et al., 2021 also identified several yield correlated taxa which matched to isolates in this studies culture collection. Subsequently, Co-inoculation studies identified 6 isolates which had a significant deleterious impact on OSR seedling biomass.

5.2 Implications and future directions

Together, the data presented in this thesis develops understanding of microbiome assembly in relation to plant host genotype, host immune hormones and functionality of rhizosphere fungi in the non-mycorrhizal crop species *B. napus* and its scientifically important relative *A. thaliana.* This is particularly important in the light of research which implicates the rhizosphere microbiome as essential to plant biology, ecology and evolution (Berendsen et al., 2012, Simon et al., 2019) and, the increasing interest in rhizosphere microbiomes as an agricultural tool (Busby et al., 2017, Chen et al., 2018, Toju et al., 2018, Trivedi et al., 2017, Yu et al., 2019). However, it should be noted that the plant species utilised in this investigation differ from the majority of vascular plants and crop species in that they are generally considered non-mycorrhizal host species (Cosme et al., 2018). Therefore, this should be taken into consideration when comparing the rhizosphere microbiome assembly and host genotype/genetics dynamics examined in this study with other crop species which have their own model organisms (Chang et al., 2016). This is especially true when examining fungal rhizosphere microbiomes, which were the focus of research in Chapters 3 and 4 of this study. Therefore, the results presented in this study may not be directly applicable to understanding plant-genotype/genetics dynamics in other

plant species. However, recent research has pointed to the blurred lines between mycorrhizal and non-mycorrhizal plant species with rudimentary associations identified in certain plants including OSR (Cosme et al., 2018). In addition, OSR is an important break crop species which is frequently rotated with wheat . Therefore, developing our understanding of rhizosphere microbiome dynamics in OSR develops our understanding of microbiome formation in soils subsequently used for growing other crop species.

The analyses presented in this thesis, emphasize the nuanced nature of genotype influences on rhizosphere microbiomes, with minimal genotype influences upon community diversity and composition but, distinct inter-taxon co-occurrence networks and indicator OTUs being identified in each genotype's microbiome. In the context of current literature, these results suggest that genotypic influences on rhizosphere microbiome assembly is genotype and context dependent. For example, other OSR genotypes with different phenotypic expressions have been shown to alter bacterial and fungal community diversity and composition (Bazghaleh et al., 2020, Taye et al., 2020). However, no community level differentiation has also been observed in a study of herbicide resistant and susceptible genotypes of OSR fungal microbiomes (Floc'h et al., 2020). In addition, edaphic, geographical and climactic factors known to influence root and rhizosphere soil microbiomes have been shown to overwhelm genotype influences in Maize, adding complexity to plant genotype – microbiome dynamics (Chen et al., 2017). Indeed, it has been observed that studies of model organisms in laboratory conditions often show host control of the microbiome to some extent with the relationship become less clear and more complex in environmentally complex field settings (Tabrett and Horton, 2020). This emphasises the need to assess genotype effects in real world field settings especially when considering crop genotype as a microbiome manipulation tool.

In addition to stressing the complex relationships between host genetics and microbial assembly, this study indicates the importance of assessing community assembly beyond analysis of diversity metrics. The unique co-occurrence networks in this study identified shifting network roles and structures for each genotype microbiome. Relationships within co-occurrence networks have previously been successfully recreated with ecologically relevant functions (Wang et al., 2017). In addition, inter-taxon interactions have been identified as key to plant health and

disease resistance in *A. thaliana* emphasising the importance of assessing inter-taxon relationships in the rhizosphere (Durán et al., 2018). Therefore, assessment of inter-taxon interactions is essential to understanding rhizosphere microbial assembly and should be included in studies of host genotype influences. However, as previously discussed there were limitations to the ability to infer or hypothesise functional capabilities of 'keystone' network hub taxa identified in this study. Therefore, several further investigations were suggested including coupling amplicon sequencing based network analysis with functional based omics techniques such as transcriptomics. This may aid in the identification of host genotypic influences beyond microbial diversity indices such as those presented in this study.

Future research which builds upon the research presented here could include several modifications including, assessment of genotypes in agricultural rotations, investigation of different genotypes and thorough assessment of potentially compounding factors. As agricultural rotation practices such as continuous cultivation are significantly associated with yield penalties and the development of deleterious microbiota in OSR (Hilton et al., 2013, Hilton et al., 2021, Knight et al., 2012), selection of OSR genotypes in rotation practices could prove useful in combating these phenomena. As the genotypes in this study were found to have significant indicator taxa and distinct co-occurrence networks, genotypic influences upon the rhizosphere may manifest in the suppression of deleterious taxa accumulating over many seasons instead of wholesale re-assembly of the microbiome at the community level. In addition, future investigations could include assessments of OSR genotypes with different phenotypic manifestations such as genotypes with resistance to the pathogenic protist *P. brassicae*.

In addition to developing understanding of plant – microbiome dynamics in terms of host genetic diversity, this study also presents data on the relationship between microbiome assembly and immune phytohormones. This study presents the first data on fungal microbiome assembly in a SA deficient plant and the first data of any microbial kingdom's assembly in response to ABA mutant plant lines. The data presented in this study indicates that SA deficiency did not significantly alter root mycobiome assembly and indicates that SA may not have a significant role in shaping overall root mycobiome assembly despite its known function in immune responses to biotrophic pathogens and systemic acquired immunity (Grant and Lamb, 2006). This

is in comparison to research which has shown significantly different bacterial microbiome assemblies in SA deficient *A. thaliana* (Lebeis et al., 2015). ABA hypersensitivity and deficiency was shown to cause a significant enrichment of Basidiomycota in the root mycobiome. However, overall community diversity was not affected. This also indicated that ABA may have a limited role in shaping overall root mycobiome assembly despite research indicating its importance in plant immune responses and the direct effects it can have on individual microbial taxa (Bari and Jones, 2009, de Torres-Zabala et al., 2007, de Torres Zabala et al., 2015, Fan et al., 2009, Sánchez-Vallet et al., 2012, Stec et al., 2016, Xu et al., 2018). Future investigations of microbiome assembly in relation to phytohormones include several avenues such as, addition of network analysis to assess community interactions, inclusion of bacterial and protist taxa in assessments and incorporation of other omics-based techniques such as transcriptomics.

Finally, the data presented in this study developed our understanding of functional interactions of ecologically relevant fungi in OSR. Co-inoculation studies identified 6 isolates which had a significant deleterious impact on OSR seedling biomass including an Uncultured Mortierella, known OSR and plant pathogens *L. biglobosa brassicae*, *Phomopsis sp.* & *Penicillum sp.* and an 'undefined saprotroph' *G. pannorum*. In addition, isolates significantly correlated with OSR yields (*T. maxilleforme* & Uncultured Microdochium) had no significant impact on biomass, indicating species specific effects on biomass and alternative mechanisms for impacting yield other than impacts on OSR biomass during the early growth stages. This identifies these taxa as priorities for further investigation of the underlying mechanisms of their potentially beneficial or deleterious relationship with OSR yield. In addition, the identification of deleterious effects from co-incubation with *G. pannorum* identifies this isolate as a priority for further investigation due to their being no record of it causing pathogenic processes in plants and due to its widespread distribution.

Together, the findings presented in this study develop our understanding of microbiome assembly in OSR and *A. thaliana* in response to host genotype and immune phytohormone production, and functionality of fungal isolates associated with OSR. This gives us insight into the ecology of rhizosphere soil and root microbiomes and develops our understanding of the mechanism involved in shaping

them. However, the goal of research such as this is to inform the development of microbiome based agricultural tools.

There are practical challenges to translating this knowledge into microbiome based agricultural manipulation tools such as transferring beneficial or protective microbiomes. As previously discussed, using host genotype/genetics as a method to cultivate a 'beneficial' microbiome for subsequent transfer poses challenges due to the apparent nuanced and situationally specific nature of host genotypes being able to influence rhizosphere microbiomes. This is especially true since it has previously been observed that studies of model organisms in laboratory conditions often show host control of the microbiome to some extent with the relationship become less clear and more complex in environmentally complex field settings (Tabrett and Horton, 2020). Therefore, careful selection of genotypes given the context of the specific agricultural field being used may be necessary to effectively use crop genotypes to transfer beneficial microbes to subsequent crops. However as previously discussed, further investigation of host genotype – microbiome dynamics is required to fully understand the underlying mechanistic processes involved in these relationships.

It is also observed in Chapter 4 that there are challenges to the direct transfer of microbiomes using soil as a medium. The process creating soil based microbial inoculants can introduce variation in the taxa represented in the inoculant, potentially changing the functionality of the transferred microbiome (Howard et al., 2017).There are also challenges to microbiome transfer through the creation of synthetic microbial communities. Cultivation-based studies are necessary to elucidate the situationally specific functionality of individual taxa or consortia in the context of the plant host (Bradáčová et al., 2019), however, the current inability to isolate most microbial taxa and the under exploration of rare and dark matter taxa compound this problem. Despite these limitations, it is still possible to create microbial consortia for microbiome transfer using currently cultivatable taxa and has been successfully carried out in glasshouse and field experiments (Yadav et al., 2017, Santoyo et al., 2021). There are many opportunities for further research in this area to expand our understanding of rhizosphere microbiomes and ultimately translate findings of studies such as this into agriculturally relevant tools and techniques for plant protection and crop improvement.

6 References

ADDY, H. D., PIERCEY, M. M. & CURRAH, R. S. 2005. Microfungal endophytes in roots. *Canadian Journal of Botany,* 83**,** 1-13.

AHDB 2018. Oilseed rape guide. 2 ed.

AHDB. 2021. *Oilseed rape disease management guidance* [Online]. AHDB. Available: https://ahdb.org.uk/osrdmg [Accessed 4th March 2021].

ALMARIO, J., JEENA, G., WUNDER, J., ZUCCARO, A., COUPLAND, G. & BUCHER, M. 2017. The root fungal microbiome of the non-mycorrhizal plant Arabis alpina and its contribution to plant P acquisition. *unpublished*

ALSTRÖM, S. 2001. Characteristics of Bacteria from Oilseed Rape in Relation to their Biocontrol Activity against Verticillium dahliae. *Journal of Phytopathology,* 149**,** 57-64.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology,* 215**,** 403-410.

AMARAL-ZETTLER, L. A., MCCLIMENT, E. A., DUCKLOW, H. W. & HUSE, S. M. 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. *PloS one,* 4**,** e6372-e6372.

ANGUS, J. F., HERWAARDEN, A. F. & HOWE, G. N. 1991. Productivity and break crop effects of winter growing oilseeds. *Austrailian Journal of Experimental Agriculture,* 31**,** 669-677.

ARENZ, B. E., HELD, B. W., JURGENS, J. A., FARRELL, R. L. & BLANCHETTE, R. A. 2006. Fungal diversity in soils and historic wood from the Ross Sea Region of Antarctica. *Soil Biology and Biochemistry,* 38**,** 3057-3064.

AVIS, P. G., DICKIE, I. A. & MUELLER, G. M. 2006. A 'dirty' business: testing the limitations of terminal restriction fragment length polymorphism (TRFLP) analysis of soil fungi. *Molecular Ecology,* 15**,** 873-882.

BADRI, D. V., CHAPARRO, J. M., ZHANG, R., SHEN, Q. & VIVANCO, J. M. 2013. Application of natural blends of phytochemicals derived from the root exudates of Arabidopsis to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *The Journal of biological chemistry,* 288**,** 4502-4512.

BALDRIAN, P. 2019. The known and the unknown in soil microbial ecology. *FEMS Microbiology Ecology,* 95.

BANERJEE, S., SCHLAEPPI, K. & VAN DER HEIJDEN, M. G. A. 2018. Keystone taxa as drivers of microbiome structure and functioning. *Nature Reviews Microbiology,* 16**,** 567-576.

BANERJEE, S., WALDER, F., BÜCHI, L., MEYER, M., HELD, A. Y., GATTINGER, A., KELLER, T., CHARLES, R. & VAN DER HEIJDEN, M. G. A. 2019. Agricultural intensification reduces microbial network complexity and the abundance of keystone taxa in roots. *The ISME Journal,* 13**,** 1722-1736.

BARI, R. & JONES, J. D. G. 2009. Role of plant hormones in plant defence responses. *Plant Molecular Biology,* 69**,** 473-488.

BASS, D., VAN DER GAST, C., THOMSON, S., NEUHAUSER, S., HILTON, S. & BENDING, G. D. 2018. Plant Rhizosphere Selection of Plasmodiophorid Lineages from Bulk Soil: The Importance of “Hidden” Diversity. *Frontiers in Microbiology,* 9.

BATTA, Y. A. 2013. Efficacy of endophytic and applied Metarhizium anisopliae (Metch.) Sorokin (Ascomycota: Hypocreales) against larvae of Plutella xylostella L. (Yponomeutidae: Lepidoptera) infesting Brassica napus plants. *Crop Protection,* 44**,** 128-134.

BAZGHALEH, N., MAMET, S. D., BELL, J. K., MOREIRA, Z. M., TAYE, Z. M., WILLIAMS, S., ARCAND, M., LAMB, E. G., SHIRTLIFFE, S., VAIL, S., SICILIANO, S. D. & HELGASON, B. 2020. An intensive multilocation temporal dataset of fungal communities in the root and rhizosphere of Brassica napus. *Data in Brief***,** 105467.

BENNETT, A., PONDER, M. M. & GARCIA-DIAZ, J. 2018. Phoma Infections: Classification, Potential Food Sources, and Its Clinical Impact. *Microorganisms,* 6**,** 58.

BERENDSEN, R. L., PIETERSE, C. M. J. & BAKKER, P. A. H. M. 2012. The rhizosphere microbiome and plant health. *Trends in Plant Science,* 17**,** 478-486.

BERGER, S., VAN WEES, S. C. M., NYBROE, O. & GROSSKINSK, D. K. 2020. Editorial: Cross-Frontier Communication: Phytohormone Functions at the Plant-Microbe Interface and Beyond. *Frontiers in Plant Science,* 11**,** 386.

BERRY, P. M. & SPINK, J. H. 2006. A physiological analysis of oilseed rape yields: Past and future. *The Journal of Agricultural Science* 144**,** 381-392.

BHUNJUN, C. S., PHILLIPS, A. J. L., JAYAWARDENA, R. S., PROMPUTTHA, I. & HYDE, K. D. 2021. Importance of Molecular Data to Identify Fungal Plant Pathogens and Guidelines for Pathogenicity Testing Based on Koch’s Postulates. *Pathogens,* 10.

BOB RUDIS, B. B., BEN MARWICK, JAN SCHULZ, ROSEN MATEV, PROPUBLICA 2017. ggalt: Extra Coordinate Systems, 'Geoms', Statistical Transformations, Scales and Fonts for 'ggplot2'. Version (0.4.0).

BOLGER, A. M., LOHSE, M. & USADEL, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics,* 30**,** 2114-20.

BOLYEN, E., RIDEOUT, J. R., DILLON, M. R., BOKULICH, N. A., ABNET, C. C., AL-GHALITH, G. A., ALEXANDER, H., ALM, E. J., ARUMUGAM, M., ASNICAR, F., BAI, Y., BISANZ, J. E., BITTINGER, K., BREJNROD, A., BRISLAWN, C. J., BROWN, C. T., CALLAHAN, B. J., CARABALLO-RODRÍGUEZ, A. M., CHASE, J., COPE, E. K., DA SILVA, R., DIENER, C., DORRESTEIN, P. C., DOUGLAS, G. M., DURALL, D. M., DUVALLET, C., EDWARDSON, C. F., ERNST, M., ESTAKI, M., FOUQUIER, J., GAUGLITZ, J. M., GIBBONS, S. M., GIBSON, D. L., GONZALEZ, A., GORLICK, K., GUO, J., HILLMANN, B., HOLMES, S., HOLSTE, H., HUTTENHOWER, C., HUTTLEY, G. A., JANSSEN, S., JARMUSCH, A. K., JIANG, L., KAEHLER, B. D., KANG, K. B., KEEFE, C. R., KEIM, P., KELLEY, S. T., KNIGHTS, D., KOESTER, I., KOSCIOLEK, T., KREPS, J., LANGILLE, M. G. I., LEE, J., LEY, R., LIU, Y.-X., LOFTFIELD, E., LOZUPONE, C., MAHER, M., MAROTZ, C., MARTIN, B. D., MCDONALD, D., MCIVER, L. J., MELNIK, A. V., METCALF, J. L., MORGAN, S. C., MORTON, J. T., NAIMEY, A. T., NAVAS-MOLINA, J. A., NOTHIAS, L. F., ORCHANIAN, S. B., PEARSON, T., PEOPLES, S. L., PETRAS, D., PREUSS, M. L., PRUESSE, E., RASMUSSEN, L. B., RIVERS, A., ROBESON, M. S., ROSENTHAL, P.,

SEGATA, N., SHAFFER, M., SHIFFER, A., SINHA, R., SONG, S. J., SPEAR, J. R., SWAFFORD, A. D., THOMPSON, L. R., TORRES, P. J., TRINH, P., TRIPATHI, A., TURNBAUGH, P. J., UL-HASAN, S., VAN DER HOOFT, J. J. J., VARGAS, F., VÁZQUEZ-BAEZA, Y., VOGTMANN, E., VON HIPPEL, M., WALTERS, W., et al. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology,* 37**,** 852-857.

BRADÁČOVÁ, K., SITTINGER, M., TIETZ, K., NEUHÄUSER, B., KANDELER, E., BERGER, N., LUDEWIG, U. & NEUMANN, G. 2019. Maize Inoculation with Microbial Consortia: Contrasting Effects on Rhizosphere Activities, Nutrient Acquisition and Early Growth in Different Soils. *Microorganisms,* 7**,** 329.

BROWN, J. K. M., BEEBY, R. & PENFIELD, S. 2019. Yield instability of winter oilseed rape modulated by early winter temperature. *Scientific Reports,* 9**,** 6953.

BRUNSON, J. C. 2020. ggalluvial: Alluvial Plots in 'ggplot2'. 0.11.3 ed. cran.

BUSBY, P. E., SOMAN, C., WAGNER, M. R., FRIESEN, M. L., KREMER, J., BENNETT, A., MORSY, M., EISEN, J. A., LEACH, J. E. & DANGL, J. L. 2017. Research priorities for harnessing plant microbiomes in sustainable agriculture. *PLOS Biology,* 15**,** e2001793.

CALLAHAN, B. J., MCMURDIE, P. J. & HOLMES, S. P. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME Journal,* 11**,** 2639-2643.

CALLAHAN, B. J., MCMURDIE, P. J., ROSEN, M. J., HAN, A. W., JOHNSON, A. J. A. & HOLMES, S. P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods,* 13**,** 581-583.

CAPORASO, J. G., KUCZYNSKI, J., STOMBAUGH, J., BITTINGER, K., BUSHMAN, F. D., COSTELLO, E. K., FIERER, N., PEÑA, A. G., GOODRICH, J. K., GORDON, J. I., HUTTLEY, G. A., KELLEY, S. T., KNIGHTS, D., KOENIG, J. E., LEY, R. E., LOZUPONE, C. A., MCDONALD, D., MUEGGE, B. D., PIRRUNG, M., REEDER, J., SEVINSKY, J. R., TURNBAUGH, P. J., WALTERS, W. A., WIDMANN, J., YATSUNENKO, T., ZANEVELD, J. & KNIGHT, R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature methods,* 7**,** 335-336.

CAPORASO, J. G., LAUBER, C. L., WALTERS, W. A., BERG-LYONS, D., HUNTLEY, J., FIERER, N., OWENS, S. M., BETLEY, J., FRASER, L., BAUER, M., GORMLEY, N., GILBERT, J. A., SMITH, G. & KNIGHT, R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal,* 6**,** 1621-1624.

CAPORASO, J. G., LAUBER, C. L., WALTERS, W. A., BERG-LYONS, D., LOZUPONE, C. A., TURNBAUGH, P. J., FIERER, N. & KNIGHT, R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences,* 108**,** 4516.

CARD, S. D., HUME, D. E., ROODI, D., MCGILL, C. R., MILLNER, J. P. & JOHNSON, R. D. 2015. Beneficial endophytic microorganisms of Brassica – A review. *Biological Control,* 90**,** 102-112.

CARRÉ, P. & POUZET, A. 2014. Rapeseed market, worldwide and in Europe. *OCL,* 21.

CARVALHAIS, L. C., DENNIS, P. G., BADRI, D. V., KIDD, B. N., VIVANCO, J. M. & SCHENK, P. M. 2015. Linking Jasmonic Acid Signaling, Root Exudates, and Rhizosphere Microbiomes. *Molecular Plant-Microbe Interactions,* 28**,** 1049-1058.

CARVALHAIS, L. C., DENNIS, P. G., BADRI, D. V., TYSON, G. W., VIVANCO, J. M. & SCHENK, P. M. 2013. Activation of the jasmonic acid plant defence pathway alters the composition of rhizosphere bacterial communities. *PLoS One,* 8**,** e56457.

CHANG, C., BOWMAN, J. L. & MEYEROWITZ, E. M. 2016. Field Guide to Plant Model Systems. *Cell,* 167**,** 325-339.

CHAPARRO, J. M., BADRI, D. V. & VIVANCO, J. M. 2014. Rhizosphere microbiome assemblage is affected by plant development. *Isme j,* 8**,** 790-803.

CHAVERRI, P., CASTLEBURY, L. A., OVERTON, B. E. & SAMUELS, G. J. 2003. Hypocrea/Trichoderma: species with conidiophore elongations and green conidia. *Mycologia,* 95**,** 1100-1140.

CHEN, L., XIN, X., ZHANG, J., REDMILE-GORDON, M. & NIE, G. 2017. Soil Characteristics Overwhelm Cultivar Effects on the Structure and Assembly of Root-Associated Microbiomes of Modern Maize. *Pedosphere*.

CHEN, M., ARATO, M., BORGHI, L., NOURI, E. & REINHARDT, D. 2018. Beneficial Services of Arbuscular Mycorrhizal Fungi – From Ecology to Application. *Frontiers in Plant Science,* 9.

COSGROVE, L., MCGEECHAN, P. L., ROBSON, G. D. & HANDLEY, P. S. 2007. Fungal communities associated with degradation of polyester polyurethane in soil. *Applied and environmental microbiology,* 73**,** 5817-5824.

COSME, M., FERNÁNDEZ, I., VAN DER HEIJDEN, M. G. A. & PIETERSE, C. M. J. 2018. Non-Mycorrhizal Plants: The Exceptions that Prove the Rule. *Trends in Plant Science,* 23.

CSARDI, G. & NEPUSZ, T. 2006. The igraph software package for complex network research. *InterJournal,* Complex Systems**,** 1695.

DE HOOG, G. S., VICENTE, V. A., NAJAFZADEH, M. J., HARRAK, M. J., BADALI, H. & SEYEDMOUSAVI, S. 2011. Waterborne Exophiala species causing disease in cold-blooded animals. *Persoonia,* 27**,** 46-72.

DE TORRES ZABALA, M., LITTLEJOHN, G., JAYARAMAN, S., STUDHOLME, D., BAILEY, T., LAWSON, T., TILLICH, M., LICHT, D., BÖLTER, B., DELFINO, L., TRUMAN, W., MANSFIELD, J., SMIRNOFF, N. & GRANT, M. 2015. Chloroplasts play a central role in plant defence and are targeted by pathogen effectors. *Nature Plants,* 1**,** 15074.

DE TORRES-ZABALA, M., TRUMAN, W., BENNETT, M. H., LAFFORGUE, G., MANSFIELD, J. W., EGEA, P. R., BÖGRE, L. & GRANT, M. 2007. Pseudomonas syringae pv.tomato hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. *The EMBO Journal,* 26**,** 1434-1443.

DOMSCH, K. H., GAMS, W. & ANDERSON, T. H. 1980. *Compendium of soil fungi. Volume 1.*, Academic Press (London) Ltd.

DU, S., DINI-ANDREOTE, F., ZHANG, N., LIANG, C., YAO, Z., ZHANG, H. & ZHANG, D. 2020. Divergent Co-occurrence Patterns and Assembly Processes Structure the Abundant and Rare Bacterial Communities in a Salt Marsh Ecosystem. *Applied and Environmental Microbiology* 86.

DUGAN, F. M. 2017. CHAPTER 4: Basidiomycota (basidiomycetes). *The Identification of Fungi: An Illustrated Introduction with Keys, Glossary, and Guide to Literature.* St Paul, MN: The American Phytopathological Society (APS) Publications.

DUNFIELD, K. E. & GERMIDA, J. J. 2001. Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified Brassica napus. *FEMS Microbiology Ecology,* 38**,** 1-9.
DUNFIELD, K. E. & GERMIDA, J. J. 2003. Seasonal Changes in the Rhizosphere Microbial Communities Associated with Field-Grown Genetically Modified Canola (Brassica napus). *Applied and Environmental Microbiology,* 69**,** 7310-7318.
DURÁN, P., THIERGART, T., GARRIDO-OTER, R., AGLER, M., KEMEN, E., SCHULZE-LEFERT, P. & HACQUARD, S. 2018. Microbial Interkingdom Interactions in Roots Promote Arabidopsis Survival. *Cell,* 175**,** 973-983.e14.
EDGAR, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics,* 26**,** 2460-1.
EDGAR, R. C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods,* 10**,** 996-8.
EDGAR, R. C., HAAS, B. J., CLEMENTE, J. C., QUINCE, C. & KNIGHT, R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics (Oxford, England),* 27**,** 2194-2200.
EDWARDS, J., JOHNSON, C., SANTOS-MEDELLÍN, C., LURIE, E., PODISHETTY, N. K., BHATNAGAR, S., EISEN, J. A. & SUNDARESAN, V. 2015. Structure, variation, and assembly of the root-associated microbiomes of rice. *Proceedings of the National Academy of Sciences of the United States of America,* 112**,** E911-E920.
EL OIRDI, M., EL RAHMAN, T. A., RIGANO, L., EL HADRAMI, A., RODRIGUEZ, M. C., DAAYF, F., VOJNOV, A. & BOUARAB, K. 2011. Botrytis cinerea Manipulates the Antagonistic Effects between Immune Pathways to Promote Disease Development in Tomato. *The Plant Cell,* 23**,** 2405-2421.
EPSTEIN, S. S. 2013. The phenomenon of microbial uncultivability. *Current Opinion in Microbiology,* 16**,** 636-642.
ERIKSSON, O. E., BARAL, H.-O., CURRAH, R. S., HANSEN, K., KURTZMAN, C. P., LÆSSØE, T. & RAMBOLD, G. 2001. Outline of Ascomycota – 2001. *Myconet,* 7**,** 1-88.
ETESAMI, H. & ALIKHANI, H. A. 2016. Rhizosphere and endorhiza of oilseed rape (Brassica napus L.) plant harbor bacteria with multifaceted beneficial effects. *Biological Control,* 94**,** 11-24.
FAN, J., HILL, L., CROOKS, C., DOERNER, P. & LAMB, C. 2009. Abscisic Acid Has a Key Role in Modulating DiversePlant-Pathogen Interactions. *Plant Physiology,* 155**,** 1750–1761.
FAO 2019a. Global Trends in Oilseed Rape Production. *In:* NATIONS, F. A. A. O. O. T. U. (ed.). FAOSTAT.
FAO 2019b. Trends in UK Oilseed Rape production (1997-2017). *In:* NATIONS, F. A. A. O. O. T. U. (ed.).
FENICE, M., SELBMANN, L., ZUCCONI, L. & ONOFRI, S. 1997. Production of extracellular enzymes by Antarctic fungal strains. *Polar Biology,* 17**,** 275-280.
FIERER, N. 2017. Embracing the unknown: disentangling the complexities of the soil microbiome. *Nature Reviews Microbiology,* 15**,** 579-590.

FLOC'H, J.-B., HAMEL, C., HARKER, K. N. & ST-ARNAUD, M. 2020. Fungal Communities of the Canola Rhizosphere: Keystone Species and Substantial Between-Year Variation of the Rhizosphere Microbiome. *Microbial Ecology*.

FOO, J. L., LING, H., LEE, Y. S. & CHANG, M. W. 2017. Microbiome engineering: Current applications and its future. *Biotechnology Journal,* 12**,** 1600099-n/a.

FRANKE-WHITTLE, I. H., MANICI, L. M., INSAM, H. & STRES, B. 2015. Rhizosphere bacteria and fungi associated with plant growth in soils of three replanted apple orchards. *Plant and Soil,* 395**,** 317-333.

GAIERO, J. R., MCCALL, C. A., THOMPSON, K. A., DAY, N. J., BEST, A. S. & DUNFIELD, K. E. 2013. Inside the root microbiome: Bacterial root endophytes and plant growth promotion. *American Journal of Botany,* 100**,** 1738-1750.

GAO, Z., KARLSSON, I., GEISEN, S., KOWALCHUK, G. & JOUSSET, A. 2019. Protists: Puppet Masters of the Rhizosphere Microbiome. *Trends in Plant Science,* 24**,** 165-176.

GKARMIRIA, K., MAHMOODA, S., EKBLADB, A., ALSTRÖMA, S., HÖGBERGA, N. & FINLAYA, R. 2017. Identifying the Active Microbiome Associated with Roots and Rhizosphere Soil of Oilseed Rape. *Applied and Environmental Microbiology* 83.

GOPAL, M. & GUPTA, A. 2016. Microbiome Selection Could Spur Next-Generation Plant Breeding Strategies. *Frontiers in Microbiology,* 7.

GRANDAUBERT, J., LOWE, R. G., SOYER, J. L., SCHOCH, C. L., VAN DE WOUW, A. P., FUDAL, I., ROBBERTSE, B., LAPALU, N., LINKS, M. G., OLLIVIER, B., LINGLIN, J., BARBE, V., MANGENOT, S., CRUAUD, C., BORHAN, H., HOWLETT, B. J., BALESDENT, M. H. & ROUXEL, T. 2014. Transposable element-assisted evolution and adaptation to host plant within the Leptosphaeria maculans-Leptosphaeria biglobosa species complex of fungal pathogens.

GRANÉR, G., PERSSON, P., MEIJER, J. & ALSTRÖM, S. 2003. A study on microbial diversity in different cultivars of Brassica napus in relation to its wilt pathogen, Verticillium longisporum. *FEMS Microbiology Letters,* 224**,** 269-276.

GRANT, M. & LAMB, C. 2006. Systemic immunity. *Curr Opin Plant Biol,* 9**,** 414-20.

GREEN, S. J., LEIGH, M. B. & NEUFELD, J. D. 2010. Denaturing Gradient Gel Electrophoresis (DGGE) for Microbial Community Analysis. *In:* TIMMIS, K. N. (ed.) *Handbook of Hydrocarbon and Lipid Microbiology.* Berlin, Heidelberg: Springer Berlin Heidelberg.

GRIFFITHS, S. M., GALAMBAO, M., ROWNTREE, J., GOODHEAD, I., HALL, J., O'BRIEN, D., ATKINSON, N. & ANTWIS, R. E. 2020. Complex associations between cross-kingdom microbial endophytes and host genotype in ash dieback disease dynamics. *Journal of Ecology,* 108.

GROSSKINSK, D. K., VAN DER GRAAFF, E. & ROITSCH, T. 2016. Regulation of Abiotic and Biotic Stress Responses by Plant Hormones *In:* COLLINGE, D. B. (ed.) *Plant Pathogen Resistance Biotechnology.* Hoboken, NJ: John Wiley & Sons, Inc.

GU, Z., GU, L., EILS, R., SCHLESNER, M. & BRORS, B. 2014. circlize implements and enhances circular visualization in R. *Bioinformatics,* 30**,** 2811-2812.

GUILLOU, L., BACHAR, D., AUDIC, S., BASS, D., BERNEY, C., BITTNER, L., BOUTTE, C., BURGAUD, G., DE VARGAS, C., DECELLE, J., DEL CAMPO, J., DOLAN, J. R.,

DUNTHORN, M., EDVARDSEN, B., HOLZMANN, M., KOOISTRA, W. H., LARA, E., LE BESCOT, N., LOGARES, R., MAHÉ, F., MASSANA, R., MONTRESOR, M., MORARD, R., NOT, F., PAWLOWSKI, J., PROBERT, I., SAUVADET, A. L., SIANO, R., STOECK, T., VAULOT, D., ZIMMERMANN, P. & CHRISTEN, R. 2013. The Protist Ribosomal Reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. *Nucleic Acids Res,* 41**,** D597-604.

GUIMERÀ, R. & AMARAL, L. 2005. Cartography of complex networks: Modules and universal roles. *Journal of statistical mechanics (Online),* 2005**,** nihpa35573.

GUSTAVO SANTOYO, C. H.-P., JULIE HERNÁNDEZ-SALMERÓN, ROCIO HERNÁNDEZ-LEÓN 2017. The role of abiotic factors modulating the plant-microbe-soil interactions: toward sustainable agriculture. A review. *Spanish Journal of Agricultural Research,* 15**,** 15.

HALE, C. 2017. *Characterisation of plant (Brassica spp.) and microbial rhizosphere functions.* PhD, University of Warwick.

HAMMER, Ø. 2019. PAST PAleontological STatistics. 3.25 ed. Natural History Museum, University of Oslo.

HARDHAM, A. R. 2011. Confocal Microscopy in Plant–Pathogen Interactions. *Plant Fungal Pathogens,* 835**,** 295-309.

HARKER, K., T O'DONOVAN, J., TURKINGTON, T., BLACKSHAW, R., Z LUPWAYI, N., G. SMITH, E., M. DOSDALL, L., HALL, L., KUTCHER, H., WILLENBORG, C., PENG, G., IRVINE, B. & MOHR, R. 2015. *Canola cultivar mixtures and rotations do not mitigate the negative impacts of continuous canola*.

HARTMANN, A., ROTHBALLER, M. & SCHMID, M. 2008. Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant and Soil,* 312**,** 7-14.

HASSANI, M. A., DURÁN, P. & HACQUARD, S. 2018. Microbial interactions within the plant holobiont. *Microbiome,* 6**,** 58.

HAWKSWORTH, D. & LÜCKING, R. 2017. Fungal Diversity Revisited: 2.2 to 3.8 Million Species. *Microbiology Spectrum,* 5.

HAYES, M. A. 2012. The Geomyces Fungi: Ecology and Distribution. *BioScience,* 62**,** 819-823.

HE, Y., REVELL, B. J., LENG, B. & FENG, Z. 2017. The Effects of Weather on Oilseed Rape (OSR) Yield in China: Future Implications of Climate Change. *Sustainability,* 9.

HERBOLD, C., PELIKAN, C., KUZYK, O., HAUSMANN, B., ANGEL, R., BERRY, D. & LOY, A. 2015. A flexible and economical barcoding approach for highly multiplexed amplicon sequencing of diverse target genes. *Frontiers in Microbiology,* 6.

HIBBETT, D. S., STAJICH, J. E. & SPATAFORA, J. W. 2013. Toward genome-enabled mycology. *Mycologia,* 105**,** 1339-1349.

HILTON, S., BENNETT, A. J., KEANE, G., BENDING, G. D., CHANDLER, D., STOBART, R. & MILLS, P. 2013. Impact of Shortened Crop Rotation of Oilseed Rape on Soil and Rhizosphere Microbial Diversity in Relation to Yield Decline. *PLOS ONE,* 8**,** e59859.

HILTON, S., PICOT, E., SCHREITER, S., BASS, D., NORMAN, K., OLIVER, A. E., MOORE, J. D., MAUCHLINE, T. H., MILLS, P. R., TEAKLE, G. R., CLARK, I. M., HIRSCH, P.

R., VAN DER GAST, C. J. & BENDING, G. D. 2021. Identification of microbial signatures linked to oilseed rape yield decline at the landscape scale. *Microbiome,* 9**,** 19.

HIRSCH, P. R. & MAUCHLINE, T. H. 2012. Who's who in the plant root microbiome? *Nature Biotechnology,* 30**,** 961.

HONG, S., BUNGE, J., LESLIN, C., JEON, S. & EPSTEIN, S. S. 2009. Polymerase chain reaction primers miss half of rRNA microbial diversity. *The ISME Journal,* 3**,** 1365-1373.

HOWARD, M. M., BELL, T. H. & KAO-KNIFFIN, J. 2017. Soil microbiome transfer method affects microbiome composition, including dominant microorganisms, in a novel environment. *FEMS microbiology letters,* 364**,** fnx092.

HOYOS-CARVAJAL, L., ORDUZ, S. & BISSETT, J. 2009. Genetic and metabolic biodiversity of Trichoderma from Colombia and adjacent neotropic regions. *Fungal Genetics and Biology,* 46**,** 615-631.

HUNTER, P. J., TEAKLE, G. R. & BENDING, G. D. 2014. Root traits and microbial community interactions in relation to phosphorus availability and acquisition, with particular reference to Brassica. *Frontiers in Plant Science,* 5**,** 27.

IHRMARK, K., BÖDEKER, I. T. M., CRUZ-MARTINEZ, K., FRIBERG, H., KUBARTOVA, A., SCHENCK, J., STRID, Y., STENLID, J., BRANDSTRÖM-DURLING, M., CLEMMENSEN, K. E. & LINDAHL, B. D. 2012. New primers to amplify the fungal ITS2 region – evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology,* 82**,** 666-677.

IRINYI, L., HOANG, M. & MEYER, W. 2017. Applying the latest next-generation sequencing technology-MinIon--to DNA barcoding based fungal identification. *Genome,* 60.

JAIN, A., BAHADUR SINGH, H. & DAS, S. 2021. Deciphering plant-microbe crosstalk through proteomics studies. *Microbiological Research,* 242.

JAIN, M., OLSEN, H. E., PATEN, B. & AKESON, M. 2016. The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. *Genome Biology,* 17.

JARI OKSANEN , F. G. B., MICHAEL FRIENDLY , ROELAND KINDT , PIERRE LEGENDRE , DAN MCGLINN , PETER R. MINCHIN , R. B. O'HARA , GAVIN L. SIMPSON , PETER SOLYMOS , M. HENRY H. STEVENS , EDUARD SZOECS , HELENE WAGNER 2019. vegan: Community Ecology Package. 2.5-5 ed.

JOCHUM, M. D., MCWILLIAMS, K. L., PIERSON, E. A. & JO, Y.-K. 2019. Host-mediated microbiome engineering (HMME) of drought tolerance in the wheat rhizosphere. *PLOS ONE,* 14**,** e0225933.

JONES, E. E., RABEENDRAN, N. & STEWART, A. 2014. Biocontrol of Sclerotinia sclerotiorum infection of cabbage by Coniothyrium minitans and Trichoderma spp. *Biocontrol Science and Technology,* 24**,** 1363-1382.

JOOS, L., BEIRINCKX, S., HAEGEMAN, A., DEBODE, J., VANDECASTEELE, B., BAEYEN, S., GOORMACHTIG, S., CLEMENT, L. & DE TENDER, C. 2020. Daring to be differential: metabarcoding analysis of soil and plant-related microbial communities using amplicon sequence variants and operational taxonomical units. *BMC Genomics,* 21**,** 733.

JOUSSET, A., BIENHOLD, C., CHATZINOTAS, A., GALLIEN, L., GOBET, A., KURM, V., KÜSEL, K., RILLIG, M. C., RIVETT, D. W., SALLES, J. F., VAN DER HEIJDEN, M. G. A., YOUSSEF, N. H., ZHANG, X., WEI, Z. & HOL, W. H. G. 2017. Where less may be more: how the rare biosphere pulls ecosystems strings. *The ISME Journal,* 11**,** 853-862.

JUMPSTART CONSORTIUM HUMAN MICROBIOME PROJECT DATA GENERATION WORKING, G. 2012. Evaluation of 16S rDNA-Based Community Profiling for Human Microbiome Research. *PLOS ONE,* 7**,** e39315.

KAMIL SLOWIKOWSKI, A. S., SEAN HUGHES, SAULIUS LUKAUSKAS, JEAN-OLIVIER IRISSON, ZHIAN N KAMVAR, THOMPSON RYAN, DERVIEUX CHRISTOPHE, YUTANI HIROAKI, PIERRE GRAMME 2019. ggrepel: Automatically Position Non-Overlapping Text Labels with 'ggplot2'. Version 0.8.1.

KASSAMBARA, A. 2018. ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.1.8. ed.

KLOEPPER, J. W., HUME, D. J., SCHER, F. M., SINGLETON, C., TIPPING, B., LALIBERTE, M., FRAULEY, K., KUTCHAW, T., SIMONSON, C. & LIFSHITZ, R. 1988. Plant growth-promoting rhizobacteria on canola (rapeseed). v. 72.

KLOEPPER, J. W., RYU, C.-M. & ZHANG, S. 2004. Induced Systemic Resistance and Promotion of Plant Growth by Bacillus spp. *Phytopathology,* 94**,** 1259-1266.

KNIEF, C. 2014. Analysis of plant microbe interactions in the era of next generation sequencing technologies. *Frontiers in Plant Science,* 5.

KNIGHT, S., KIGHTLEY, S., BINGHAM, I., HOAD, S., LANG, B., PHILPOTT, H., STOBART, R., THOMAS, J., BARNES, A. & BALL, B. 2012. Project Report No. 502. HGCA.

KÕLJALG, U., NILSSON, R. H., ABARENKOV, K., TEDERSOO, L., TAYLOR, A. F., BAHRAM, M., BATES, S. T., BRUNS, T. D., BENGTSSON-PALME, J., CALLAGHAN, T. M., DOUGLAS, B., DRENKHAN, T., EBERHARDT, U., DUEÑAS, M., GREBENC, T., GRIFFITH, G. W., HARTMANN, M., KIRK, P. M., KOHOUT, P., LARSSON, E., LINDAHL, B. D., LÜCKING, R., MARTÍN, M. P., MATHENY, P. B., NGUYEN, N. H., NISKANEN, T., OJA, J., PEAY, K. G., PEINTNER, U., PETERSON, M., PÕLDMAA, K., SAAG, L., SAAR, I., SCHÜSSLE, A., SCOTT, J. A., SENÉS, C., SMITH, M. E., SUIJA, A., TAYLOR, D. L., TELLERIA, M. T., WEISS, M. & LARSSON, K. H. 2013. Towards a unified paradigm for sequence-based identification of fungi. *Mol Ecol,* 22**,** 5271-7.

KORENBLUM, E., DONG, Y., SZYMANSKI, J., PANDA, S., JOZWIAK, A., MASSALHA, H., MEIR, S., ROGACHEV, I. & AHARONI, A. 2020. Rhizosphere microbiome mediates systemic root metabolite exudation by root-to-root signaling. *Proceedings of the National Academy of Sciences,* 117**,** 3874-3883.

KOZICH, J. J., WESTCOTT, S. L., BAXTER, N. T., HIGHLANDER, S. K. & SCHLOSS, P. D. 2013. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Applied and Environmental Microbiology,* 79**,** 5112-5120.

KUTSCHERA, U. & KHANNA, R. 2016. Plant gnotobiology: Epiphytic microbes and sustainable agriculture. *Plant Signaling & Behavior,* 11**,** e1256529.

LAKSHMANAN, V., SELVARAJ, G. & BAIS, H. P. 2014. Functional Soil Microbiome: Belowground Solutions to an Aboveground Problem. *Plant Physiology,* 166**,** 689-700.

LANGILLE, M. G. I., ZANEVELD, J., CAPORASO, J. G., MCDONALD, D., KNIGHTS, D., REYES, J. A., CLEMENTE, J. C., BURKEPILE, D. E., VEGA THURBER, R. L., KNIGHT, R., BEIKO, R. G. & HUTTENHOWER, C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology,* 31**,** 814-821.

LAREEN, A., BURTON, F. & SCHÄFER, P. 2016. Plant root-microbe communication in shaping root microbiomes. *Plant molecular biology,* 90**,** 575-587.

LAU, J. A. & LENNON, J. T. 2012. Rapid responses of soil microorganisms improve plant fitness in novel environments. *Proceedings of the National Academy of Sciences,* 109**,** 14058-14062.

LAY, C.-Y., BELL, T. H., HAMEL, C., HARKER, K. N., MOHR, R., GREER, C. W., YERGEAU, É. & ST-ARNAUD, M. 2018a. Canola Root–Associated Microbiomes in the Canadian Prairies. *Frontiers in Microbiology,* 9.

LAY, C.-Y., HAMEL, C. & ST-ARNAUD, M. 2018b. Taxonomy and pathogenicity of Olpidium brassicae and its allied species. *Fungal biology,* 122**,** 837-846.

LEBEIS, S. L., PAREDES, S. H., LUNDBERG, D. S., BREAKFIELD, N., GEHRING, J., MCDONALD, M., MALFATTI, S., GLAVINA DEL RIO, T., JONES, C. D., TRINGE, S. G. & DANGL, J. L. 2015. Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science,* 349**,** 860-864.

LIN PEDERSEN, T. 2019. ggforce: Accelerating 'ggplot2' Version (0.3.1.9000).

LIN, W., WU, L., LIN, S., ZHANG, A., ZHOU, M., LIN, R., WANG, H., CHEN, J., ZHANG, Z. & LIN, R. 2013. Metaproteomic analysis of ratoon sugarcane rhizospheric soil. *BMC Microbiology,* 13**,** 135.

LINDSTRÖM, S., ROWE, O., TIMONEN, S., SUNDSTRÖM, L. & JOHANSSON, H. 2018. Trends in bacterial and fungal communities in ant nests observed with Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and Next Generation Sequencing (NGS) techniques—validity and compatibility in ecological studies. *PeerJ,* 6**,** e5289.

LIU, H., CARVALHAIS, L. C., SCHENK, P. M. & DENNIS, P. G. 2017. Effects of jasmonic acid signalling on the wheat microbiome differ between body sites. *Sci Rep,* 7**,** 41766.

LIU, W. T., MARSH, T. L., CHENG, H. & FORNEY, L. J. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol,* 63**,** 4516-22.

LIU, Z., DESANTIS, T. Z., ANDERSEN, G. L. & KNIGHT, R. 2008. Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. *Nucleic Acids Research,* 36**,** e120-e120.

LLOYD, K. G., STEEN, A. D., LADAU, J., YIN, J., CROSBY, L. & NEUFELD, J. D. 2018. Phylogenetically Novel Uncultured Microbial Cells Dominate Earth Microbiomes. *mSystems,* 3**,** e00055-18.

LUCACIU, R., PELIKAN, C., GERNER, S. M., ZIOUTIS, C., KÖSTLBACHER, S., MARX, H., HERBOLD, C. W., SCHMIDT, H. & RATTEI, T. 2019. A Bioinformatics Guide to Plant Microbiome Analysis. *Frontiers in Plant Science,* 10.

LUCAS, M., BALBÍN-SUÁREZ, A., SMALLA, K. & VETTERLEIN, D. 2018. Root growth, function and rhizosphere microbiome analyses show local rather than

systemic effects in apple plant response to replant disease soil. *PLOS ONE,* 13**,** e0204922.
MA, L.-J., GEISER, D. M., PROCTOR, R. H., ROONEY, A. P., O'DONNELL, K., TRAIL, F., GARDINER, D. M., MANNERS, J. M. & KAZAN, K. 2013. Fusarium Pathogenomics. *Annual Review of Microbiology,* 67**,** 399-416.
MACIÁ-VICENTE, J. G., GLYNOU, K. & PIEPENBRING, M. 2016. A new species of Exophiala associated with roots. *Mycological Progress,* 15**,** 1-12.
MANZOTTI, A., BERGNA, A., BUROW, M., JØRGENSEN, H. J. L., CERNAVA, T., BERG, G., COLLINGE, D. B. & JENSEN, B. 2020. Insights into the community structure and lifestyle of the fungal root endophytes of tomato by combining amplicon sequencing and isolation approaches with phytohormone profiling. *FEMS Microbiol Ecol,* 96.
MARTIN, F. N. & LOPER, J. E. 1999. Soilborne Plant Diseases Caused by Pythium spp.: Ecology, Epidemiology, and Prospects for Biological Control. *Critical Reviews in Plant Sciences,* 18**,** 111-181.
MARTÍNEZ-GARCIA, L. B., OCHOA-HUESO, R., MANRIQUE, E. & PUGNAIRE, F. I. 2015. Different mycorrhizal fungal strains determine plant community responseto nitrogen and water availability. *Journal of Plant Nutrition and Soil Science,* 178**,** 146-154.
MARTINS, S. J., ROCHA, G. A., DE MELO, H. C., DE CASTRO GEORG, R., ULHÔA, C. J., DE CAMPOS DIANESE, É., OSHIQUIRI, L. H., DA CUNHA, M. G., DA ROCHA, M. R., DE ARAÚJO, L. G., VAZ, K. S. & DUNLAP, C. A. 2018. Plant-associated bacteria mitigate drought stress in soybean. *Environ Sci Pollut Res Int,* 25**,** 13676-13686.
MCCARTHY, C. G. P. & FITZPATRICK, D. A. 2019. Pan-genome analyses of model fungal species. *Microbial Genomics,* 5.
MCDONALD, D., PRICE, M. N., GOODRICH, J., NAWROCKI, E. P., DESANTIS, T. Z., PROBST, A., ANDERSEN, G. L., KNIGHT, R. & HUGENHOLTZ, P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *Isme j,* 6**,** 610-8.
MCMURDIE, P. J. & HOLMES, S. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE,* 8**,** e61217.
MCVETTY, P. B. E., MIETKIEWSKA, E., OMONOV, T., CURTIS, J., TAYLOR, D. C. & WESELAKE, R. J. 2016. Brassica spp. Oils. *Industrial oil crops* Academic Press.
MICALLEF, S. A., CHANNER, S., SHIARIS, M. P. & COLÓN-CARMONA, A. 2009. Plant age and genotype impact the progression of bacterial community succession in the Arabidopsis rhizosphere. *Plant signaling & behavior,* 4**,** 777-780.
MIZUBUTI, E. S. G. 2019. Special issue on white mold - Sclerotinia sclerotiorum. *Tropical Plant Pathology,* 44**,** 1-2.
MORGAN, H. H., DU TOIT, M. & SETATI, M. E. 2017. The Grapevine and Wine Microbiome: Insights from High-Throughput Amplicon Sequencing. *Frontiers in Microbiology,* 8.
MOUGEL, C., LEBRETON, L., GUILLERM-ERCKELBOUDT, A.-Y., GAZENGEL, K., LINGLIN, J., OURRY, M., GLORY, P., SARNIGUET, A., DAVAL, S. & MANZANARES-DAULEUX, M. J. 2018. Temporal dynamics of bacterial and

fungal communities during the infection of Brassica rapa roots by the protist Plasmodiophora brassicae. *bioRxiv*.

MUNKVOLD, L., KJØLLER, R., VESTBERG, M., ROSENDAHL, S. & JAKOBSEN, I. 2004. High functional diversity within species of arbuscular mycorrhizal fungi. *New Phytologist,* 164**,** 357-364.

MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology,* 59**,** 695-700.

NA, X., CAO, X., MA, C., MA, S., XU, P., LIU, S., WANG, J., WANG, H., CHEN, L. & QIAO, Z. 2019. Plant Stage, Not Drought Stress, Determines the Effect of Cultivars on Bacterial Community Diversity in the Rhizosphere of Broomcorn Millet (Panicum miliaceum L.). *Frontiers in Microbiology,* 10.

NAGAHARU, U. 1935. Genome Analysis in Brassica with Special Reference to the Experimental Formation of B. Napus and Peculiar Mode of Fertilization. *Japanese Journal of Botany,* 7**,** 389-452.

NALLANCHAKRAVARTHULA, S., MAHMOOD, S., ALSTRÖM, S. & FINLAY, R. D. 2014. Influence of Soil Type, Cultivar and Verticillium dahliae on the Structure of the Root and Rhizosphere Soil Fungal Microbiome of Strawberry. *PLOS ONE,* 9**,** e111455.

NAWRATH, C. & MÉTRAUX, J.-P. 1999. Salicylic Acid Induction–Deficient Mutants of Arabidopsis Express PR-2 and PR-5 and Accumulate High Levels of Camalexin after Pathogen Inoculation. *The Plant Cell,* 11**,** 1393-1404.

NGUYEN, N.-P., WARNOW, T., POP, M. & WHITE, B. 2016a. A perspective on 16S rRNA operational taxonomic unit clustering using sequence similarity. *npj Biofilms and Microbiomes,* 2**,** 16004.

NGUYEN, N. H., SONG, Z., BATES, S. T., BRANCO, S., TEDERSOO, L., MENKE, J., SCHILLING, J. S. & KENNEDY, P. G. 2016b. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecology,* 20**,** 241-248.

O. WILKE, C. 2019. cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'. version (1.0.0)

OGLE, D., WHEELER, P. & DINNO, A. 2021. FSA: Fisheries Stock Analysis. R package version 0.8.32 ed.

OLESEN, J. M., BASCOMPTE, J., DUPONT, Y. L. & JORDANO, P. 2007. The modularity of pollination networks. *Proceedings of the National Academy of Sciences,* 104**,** 19891-19896.

OZIMEK, E. & HANAKA, A. 2021. MortierellaSpecies as the Plant Growth-Promoting FungiPresent in the Agricultural Soils. *Agriculture,* 11.

PAUVERT, C., VALLANCE, J., DELIÈRE, L., BUÉE, M. & VACHER, C. 2019. Microbial networks inferred from metabarcoding data lack replicability: consequences for next-generation biomonitoring. *bioRxiv***,** 642199.

PEAY, K. G. 2014. Back to the future: natural history and the way forward in modern fungal ecology. *Fungal Ecology,* 12**,** 4-9.

PIETERSE, C. M. J., VAN DER DOES, D., ZAMIOUDIS, C., LEON-REYES, A. & VAN WEES, S. C. M. 2012. Hormonal Modulation of Plant Immunity. *Annual Review of Cell and Developmental Biology,* 28**,** 489-521.

POVEDA, J., HERMOSA, R., MONTE, E. & NICOLÁS, C. 2019. Trichoderma harzianum favours the access of arbuscular mycorrhizal fungi to non-host Brassicaceae roots and increases plant productivity. *Scientific Reports,* 9**,** 11650.

POZO, M. J., ZABALGOGEAZCOA, I., VAZQUEZ DE ALDANA, B. R. & A., M.-M. 2021. Untapping the potential of plant mycobiomes for applications in agriculture. *Current Opinion in Plant Biology,* 60.

QUAST, C., PRUESSE, E., YILMAZ, P., GERKEN, J., SCHWEER, T., YARZA, P., PEPLIES, J. & GLÖCKNER, F. O. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res,* 41**,** D590-6.

QUIZA, L., ST-ARNAUD, M. & YERGEAU, E. 2015. Harnessing phytomicrobiome signaling for rhizosphere microbiome engineering. *Frontiers in Plant Science,* 6**,** 507.

R CORE TEAM 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

R CORE TEAM 2018. R: A language and environment for statistical Computing.

RAMIREZ, K. S., KNIGHT, C. G., DE HOLLANDER, M., BREARLEY, F. Q., CONSTANTINIDES, B., COTTON, A., CREER, S., CROWTHER, T. W., DAVISON, J., DELGADO-BAQUERIZO, M., DORREPAAL, E., ELLIOTT, D. R., FOX, G., GRIFFITHS, R. I., HALE, C., HARTMAN, K., HOULDEN, A., JONES, D. L., KRAB, E. J., MAESTRE, F. T., MCGUIRE, K. L., MONTEUX, S., ORR, C. H., VAN DER PUTTEN, W. H., ROBERTS, I. S., ROBINSON, D. A., ROCCA, J. D., ROWNTREE, J., SCHLAEPPI, K., SHEPHERD, M., SINGH, B. K., STRAATHOF, A. L., BHATNAGAR, J. M., THION, C., VAN DER HEIJDEN, M. G. A. & DE VRIES, F. T. 2018. Detecting macroecological patterns in bacterial communities across independent studies of global soils. *Nature Microbiology,* 3**,** 189-196.

RAO, C., COYTE, K. Z., BAINTER, W., GEHA, R. S., MARTIN, C. R. & RAKOFF-NAHOUM, S. 2020. Multi-kingdom quantitation reveals distinct ecological drivers of predictable early-life microbiome assembly. *bioRxiv***,** 2020.03.02.970061.

RATHORE, R., DOWLING, D. N., FORRISTAL, P. D., SPINK, J., COTTER, P. D., BULGARELLI, D. & GERMAINE, K. J. 2017. Crop Establishment Practices Are a Driver of the Plant Microbiota in Winter Oilseed Rape (Brassica napus). *Frontiers in microbiology* [Online], 8. [Accessed 2017].

RAUSCH, P., RÜHLEMANN, M., HERMES, B. M., DOMS, S., DAGAN, T., DIERKING, K., DOMIN, H., FRAUNE, S., VON FRIELING, J., HENTSCHEL, U., HEINSEN, F.-A., HÖPPNER, M., JAHN, M. T., JASPERS, C., KISSOYAN, K. A. B., LANGFELDT, D., REHMAN, A., REUSCH, T. B. H., ROEDER, T., SCHMITZ, R. A., SCHULENBURG, H., SOLUCH, R., SOMMER, F., STUKENBROCK, E., WEILAND-BRÄUER, N., ROSENSTIEL, P., FRANKE, A., BOSCH, T. & BAINES, J. F. 2019. Comparative analysis of amplicon and metagenomic sequencing methods reveals key features in the evolution of animal metaorganisms. *Microbiome,* 7**,** 133.

ROBERTS, D. W. 2019. labdsv: Ordination and Multivariate Analysis for Ecology. 2.0-1 ed.

ROCHEFORT, A., BRIAND, M., MARAIS, C., WAGNER, M.-H., LAPERCHE, A., VALLÉE, P., BARRET, M. & SARNIGUET, A. 2019. Influence of Environment and Host Plant Genotype on the Structure and Diversity of the Brassica napus Seed Microbiota. *Phytobiomes Journal,* 3**,** 326-336.

RONDANINI, D., GÓMEZ, N., AGOSTIB, M. & MIRALLES, D. 2012. Global trends of rapeseed grain yield stability and rapeseed-to-wheat yield ratio in the last four decades. *European Journal of Agronomy*.

ROSSMANN, M., PÉREZ-JARAMILLO, J. E., KAVAMURA, V. N., CHIARAMONTE, J. B., DUMACK, K., FIORE-DONNO, A. M., MENDES, L. W., FERREIRA, M. M. C., BONKOWSKI, M., RAAIJMAKERS, J. M., MAUCHLINE, T. H. & MENDES, R. 2020. Multitrophic interactions in the rhizosphere microbiome of wheat: from bacteria and fungi to protists. *FEMS Microbiology Ecology,* 96.

RSTUDIO TEAM 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA version 1.1.456.

RYBAKOVA, D., MANCINELLI, R., WIKSTRÖM, M., BIRCH-JENSEN, A.-S., POSTMA, J., EHLERS, R.-U., GOERTZ, S. & BERG, G. 2017. The structure of the Brassica napus seed microbiome is cultivar-dependent and affects the interactions of symbionts and pathogens. *Microbiome,* 5**,** 104.

SAAVEDRA, S., STOUFFER, D. B., UZZI, B. & BASCOMPTE, J. 2011. Strong contributors to network persistence are the most vulnerable to extinction. *Nature,* 478**,** 233-235.

SACHS, J. L., RUSSELL, J. E. & HOLLOWELL, A. C. 2011. Evolutionary Instability of Symbiotic Function in Bradyrhizobium japonicum. *PLOS ONE,* 6.

SÁNCHEZ-VALLET, A., LÓPEZ, G., RAMOS, B., DELGADO-CEREZO, M., RIVIERE, M.-P., LLORENTE, F., FERNÁNDEZ, P. V., MIEDES, E., ESTEVEZ, J. M., GRANT, M. & MOLINA, A. 2012. Disruption of Abscisic Acid Signaling ConstitutivelyActivates Arabidopsis Resistance to the NecrotrophicFungusPlectosphaerella cucumerina. *Plant Physiology,* 160**,** 2109–2124.

SANTOYO, G., GUZMÁN-GUZMÁN, P., PARRA-COTA, F. I., SANTOS-VILLALOBOS, S. D. L., OROZCO-MOSQUEDA, M. D. C. & GLICK, B. R. 2021. Plant Growth Stimulation by Microbial Consortia. *Agronomy,* 11**,** 219.

SARANGTHEM INDIRA DEVI, P. M. 2015. Plant-Endophyte Interaction and its Unrelenting Contribution to Plant Health *In:* ARORA, N. K. (ed.) *Plant Microbe Symbiosis: Applied Facets* Springer India

SCHLATTER, D. C., HANSEN, J. C., SCHILLINGER, W. F., SULLIVAN, T. S. & PAULITZ, T. C. 2019. Common and unique rhizosphere microbial communities of wheat and canola in a semiarid Mediterranean environment. *Applied Soil Ecology,* 144**,** 170-181.

SCHLEMPER, T. R., LEITE, M. F. A., LUCHETA, A. R., SHIMELS, M., BOUWMEESTER, H. J., VAN VEEN, J. A. & KURAMAE, E. E. 2017. Rhizobacterial community structure differences among sorghum cultivars in different growth stages and soils. *FEMS Microbiology Ecology,* 93.

SCHLOSS, P. D. 2010. The Effects of Alignment Quality, Distance Calculation Method, Sequence Filtering, and Region on the Analysis of 16S rRNA Gene-Based Studies. *PLOS Computational Biology,* 6**,** e1000844.

SCHLOSS, P. D. & MCBAIN, A. J. 2020. Reintroducing mothur: 10 Years Later. *Applied and Environmental Microbiology,* 86**,** e02343-19.
SCHLOSS, P. D., WESTCOTT, S. L., RYABIN, T., HALL, J. R., HARTMANN, M., HOLLISTER, E. B., LESNIEWSKI, R. A., OAKLEY, B. B., PARKS, D. H., ROBINSON, C. J., SAHL, J. W., STRES, B., THALLINGER, G. G., HORN, D. J. V. & WEBER, C. F. 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology,* 75**,** 7537-7541.
SELOSSE, M. A., VOHNÍK, M. & CHAUVET, E. 2008. Out of the rivers: are some aquatic hyphomycetes plant endophytes? *New Phytol,* 178**,** 3-7.
SHAKYA, M., LO, C.-C. & CHAIN, P. S. G. 2019. Advances and Challenges in Metatranscriptomic Analysis. *Frontiers in Genetics,* 10.
SHARPTON, T. J. 2014. An introduction to the analysis of shotgun metagenomic data. *Frontiers in Plant Science,* 5.
SICILIANO, S. D. & GERMIDA, J. J. 1999. Taxonomic diversity of bacteria associated with the roots of field-grown transgenic Brassica napus cv. Quest, compared to the non-transgenic B. napus cv. Excel and B. rapa cv. Parkland,. *FEMS Microbiology Ecology,* 29**,** 263-272.
SIMON, J.-C., MARCHESI, J. R., MOUGEL, C. & SELOSSE, M.-A. 2019. Host-microbiota interactions: from holobiont theory to analysis. *Microbiome,* 7**,** 5.
SOGIN, M. L., MORRISON, H. G., HUBER, J. A., WELCH, D. M., HUSE, S. M., NEAL, P. R., ARRIETA, J. M. & HERNDL, G. J. 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences,* 103**,** 12115-12120.
SPENCE, C., ALFF, E., JOHNSON, C., RAMOS, C., DONOFRIO, N., SUNDARESAN, V. & BAIS, H. 2014. Natural rice rhizospheric microbes suppress rice blast infections. *BMC Plant Biology,* 14**,** 130-130.
STEC, N., BANASIAK, J. & JASIŃSKI, M. 2016. Abscisic acid - an overlooked player in plant-microbe symbioses formation? *Acta Biochim Pol,* 63**,** 53-58.
STOECK, T., BASS, D., NEBEL, M., CHRISTEN, R., JONES, M. D. M., BREINER, H.-W. & RICHARDS, T. A. 2010. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. *Molecular Ecology,* 19**,** 21-31.
SUN, C., SHAO, Y., VAHABI, K., LU, J., BHATTACHARYA, S., DONG, S., YEH, K. W., SHERAMETI, I., LOU, B., BALDWIN, I. T. & OELMÜLLER, R. 2014. The beneficial fungus Piriformospora indica protects Arabidopsis from Verticillium dahliae infection by downregulation plant defense responses. *BMC Plant Biol,* 14**,** 268.
TABRETT, A. & HORTON, M. W. 2020. The influence of host genetics on the microbiome. *F1000Research,* 9**,** F1000 Faculty Rev-84.
TAMM, H. & PÕLDMMA, K. 2013. Diversity, host associations, and phylogeography of temperate aurofusarin-producing Hypomyces/Cladobotryum including causal agents of cobweb disease of cultivated mushrooms. *Fungal Biology,* 117**,** 348-367.
TANG, M. H. A. Y. 2016. ggfortify: Data Visualization Tools for Statistical Analysis Results. .

TAYE, Z. M., HELGASON, B. L., BELL, J. K., NORRIS, C. E., VAIL, S., ROBINSON, S. J., PARKIN, I. A. P., ARCAND, M., MAMET, S., LINKS, M. G., DOWHY, T., SICILIANO, S. & LAMB, E. G. 2020. Core and Differentially Abundant Bacterial Taxa in the Rhizosphere of Field Grown Brassica napus Genotypes: Implications for Canola Breeding. *Frontiers in Microbiology,* 10.

THÉBAULT, E. & FONTAINE, C. 2010. Stability of Ecological Communities and the Architecture of Mutualistic and Trophic Networks. *Science,* 329**,** 853.

THOMAS, C. L., GRAHAM, N. S., HAYDEN, R., MEACHAM, M. C., NEUGEBAUER, K., NIGHTINGALE, M., DUPUY, L. X., HAMMOND, J. P., WHITE, P. J. & BROADLEY, M. R. 2016. High-throughput phenotyping (HTP) identifies seedling root traits linked to variation in seed yield and nutrient capture in field-grown oilseed rape (Brassica napus L.). *Annals of Botany,* 118**,** 655-665.

TIAN, T., REVERDY, A., SHE, Q., SUN, B. & CHAI, Y. 2020. The role of rhizodeposits in shaping rhizomicrobiome. *Environmental Microbiology Reports,* 12**,** 160-172.

TOJU, H., PEAY, K. G., YAMAMICHI, M., NARISAWA, K., HIRUMA, K., NAITO, K., FUKUDA, S., USHIO, M., NAKAOKA, S., ONODA, Y., YOSHIDA, K., SCHLAEPPI, K., BAI, Y., SUGIURA, R., ICHIHASHI, Y., MINAMISAWA, K. & KIERS, E. T. 2018. Core microbiomes for sustainable agroecosystems. *Nature Plants,* 4**,** 247-257.

TRIVEDI, P., SCHENK, P. M., WALLENSTEIN, M. D. & SINGH, B. K. 2017. Tiny Microbes, Big Yields: enhancing food crop production with biological solutions. *Microb Biotechnol,* 10**,** 999-1003.

UECKER, F. A. 1988. *A world list of Phomopsis names with notes on nomen-clature, morphology, and biology.*

UNITE-COMMUNITY 2019. UNITE QIIME release for Fungi. (8.0) 18.11.2018 ed.: UNITE Community.

ÚRBEZ-TORRES, J. R., PEDUTO, F., SMITH, R. J. & GUBLER, W. D. 2013. Phomopsis Dieback: A Grapevine Trunk Disease Caused by Phomopsis viticolain California. *Plant Disease***,** 1571-1579.

VAN DE PEER, Y., CHAPELLE, S. & DE WACHTER, R. 1996. A quantitative map of nucleotide substitution rates in bacterial rRNA. *Nucleic Acids Res,* 24**,** 3381-91.

VANGELISTI, A., NATALI, L., BERNARDI, R., SBRANA, C., TURRINI, A., HASSANI-PAK, K., CAVALLINI, A., GIOVANNETTI, M. & GIORDANI, T. 2018. Transcriptome changes induced by arbuscular mycorrhizal fungi in sunflower (Helianthus annuus L.) roots. *Scientific Reports,* 8.

VESTHEIM, H. & JARMAN, S. N. 2008. Blocking primers to enhance PCR amplification of rare sequences in mixed samples - a case study on prey DNA in Antarctic krill stomachs. *Frontiers in zoology,* 5**,** 12-12.

VOORRIPS, R. E. 1992. Root hair infection by Plasmodiophora brassicae in clubrootresistant and susceptible genotypes of Brassica oleracea, B. rapa and B. napus. *Netherlands Journal of Plant Pathology,* 98**,** 361-368.

WAGNER, C., ANKE, H., BESL, H. & STERNER, O. 1995. Flavipucine and Brunnescin, Two Antibiotics from Cultures of theMycophilic Fungus Cladobotryum rubrobrunnescens. *Zeitschrift für Naturforschung* 50**,** 358-364.

WAGNER, C., ANKE, H. & STERNER, O. 1998. Rubrobramide, a Cytotoxic and Phytotoxic Metabolite from Cladobotryum rubrobrunnescens. *Journal of Natural Products* 61**,** 501-502.

WAGNER, M. R., LUNDBERG, D. S., DEL RIO, T. G., TRINGE, S. G., DANGL, J. L. & MITCHELL-OLDS, T. 2016. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nature communications,* 7**,** 12151-12151.

WANG, H., WEI, Z., MEI, L., GU, J., YIN, S., FAUST, K., RAES, J., DENG, Y., WANG, Y., SHEN, Q. & YIN, S. 2017. Combined use of network inference tools identifies ecologically meaningful bacterial associations in a paddy soil. *Soil Biology and Biochemistry,* 105**,** 227-235.

WANG, J., LI, R., ZHANG, H., WEI, G. & LI, Z. 2020. Beneficial bacteria activate nutrients and promote wheat growth under conditions of reduced fertilizer application. *BMC Microbiology,* 20**,** 38.

WATKINSON, S. C. 2008. *Basidiomycota,* Wiley Online Library.

WATTS, S. C., RITCHIE, S. C., INOUYE, M. & HOLT, K. E. 2019. FastSpar: rapid and scalable correlation estimation for compositional data. *Bioinformatics,* 35**,** 1064–1066.

WESTCOTT, S. L. & SCHLOSS, P. D. 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. *PeerJ,* 3**,** e1487.

WHITE, J. F., KINGSLEY, K. L., ZHANG, Q., VERMA, R., OBI, N., DVINSKIKH, S., ELMORE, M. T., VERMA, S. K., GOND, S. K. & KOWALSKI, K. P. 2019. Review: Endophytic microbes and their potential applications in crop management. *Pest management science,* 75**,** 2558-2565.

WHITE, T. J., BURNS, T., LEE, S. & TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* INNIS, M. A., GELFAND, D. H., SNINSKY, J. J. & WHITE, T. J. (eds.) *PCR Protocols: A Guide to Methods and Applications.* Academic Press

WHITFIELD, W. A. D. 1974. The Soils of the National Vegetable Research Station, Wellesbourne. *Soil Survey of England and Wales.*

WICKHAM, H. 2016. *ggplot2: Elegant Graphics for Data Analysis*, Springer-Verlag New York.

WOESE, C. R. 1987. Bacterial Evolution. *Microbiological Reviews,* 51**,** 221-271.

WU, B., HUSSAIN, M., ZHANG, W., STADLER, M., LIU, X. & XIANG, M. 2019. Current insights into fungal species diversity and perspective on naming the environmental DNA sequences of fungi. *Mycology,* 10**,** 127-140.

WYLIE, K. M., TRUTY, R. M., SHARPTON, T. J., MIHINDUKULASURIYA, K. A., ZHOU, Y., GAO, H., SODERGREN, E., WEINSTOCK, G. M. & POLLARD, K. S. 2012. Novel Bacterial Taxa in the Human Microbiome. *PLOS ONE,* 7**,** e35294.

XIONG, W., SONG, Y., YANG, K., GU, Y., WEI, Z., KOWALCHUK, G. A., XU, Y., JOUSSET, A., SHEN, Q. & GEISEN, S. 2020. Rhizosphere protists are key determinants of plant health. *Microbiome,* 8**,** 27.

XU, G., YANG, S., MENG, L. & WANG, B.-G. 2018. The plant hormone abscisic acid regulates the growth and metabolism of endophytic fungus Aspergillus nidulans. *Scientific Reports,* 8**,** 6504.

YADAV, G., VISHWAKARMA, K., SHARMA, S., KUMAR, V., UPADHYAY, N., KUMAR, N., VERMA, R. K., MISHRA, R., TRIPATHI, D. K. & UPADHYAY, R. G. 2017. Emerging Significance of Rhizospheric Probiotics and Its Impact on Plant Health: Current Perspective Towards Sustainable Agriculture. *In:* KUMAR, V., KUMAR, M., SHARMA, S. & PRASAD, R. (eds.) *Probiotics and Plant Health.* Springer.

YU, K., PIETERSE, C. M. J., BAKKER, P. A. H. M. & BERENDSEN, R. L. 2019. Beneficial microbes going underground of root immunity. *Plant, Cell & Environment,* 42**,** 2860-2870.

YU, P. & HOCHHOLDINGER, F. 2018. The Role of Host Genetic Signatures on Root–Microbe Interactions in the Rhizosphere and Endosphere. *Frontiers in Plant Science,* 9.

YUAN TANG, M. H., AND WENXUAN LI 2016. ggfortify: Unified Interface to Visualize Statistical Result of Popular R Packages. *The R Journal* 8.2 478-489.

YURGEL, S. N., NEARING, J. T., DOUGLAS, G. M. & LANGILLE, M. G. I. 2019. Metagenomic Functional Shifts to Plant Induced Environmental Changes. *Frontiers in Microbiology,* 10.

ZAMKOVAYA, T., FOSTER, J. S., DE CRÉCY-LAGARD, V. & CONESA, A. 2021. A network approach to elucidate and prioritize microbial dark matter in microbial communities. *The ISME Journal,* 15**,** 228-244.

ZHANG, Q., ZHANG, J., YANG, L., ZHANG, L., JIANG, D., CHEN, W. & LI, G. 2014. Diversity and biocontrol potential of endophytic fungi in Brassica napus. *Biological Control,* 72**,** 98-108.

ZHAO, Y., GAO, Z., TIAN, B., BI, K., CHEN, T., LIU, H., XIE, J., CHENG, J., FU, Y. & JIANG, D. 2017. Endosphere microbiome comparison between symptomatic and asymptomatic roots of Brassica napus infected with Plasmodiophora brassicae. *PLOS ONE,* 12**,** e0185907.

ZHENG, X., KOOPMANN, B., ULBER, B. & VON TIEDEMANN, A. 2020. A Global Survey on Diseases and Pests in Oilseed Rape—Current Challenges and Innovative Strategies of Control. *Frontiers in Agronomy,* 2.

ZHONG, Y., HU, J., XIA, Q., ZHANG, S., LI, X., PAN, X., ZHAO, R., WANG, R., YAN, W., SHANGGUAN, Z., HU, F., YANG, C. & WANG, W. 2020. Soil microbial mechanisms promoting ultrahigh rice yield. *Soil Biology and Biochemistry,* 143**,** 107741.

7 Appendix

Table A1 Nutrient profiles of growth media and Wellesbourne soil, genotype conditioned soils and microbiome transfer inoculants. *(-) indicates samples awaiting return of nutrient profiling results from Lancrop Laboratories.*

Growth medium	pH	Nitrate N (mg/kg)	Phosphorus (ppm)	Potassium (ppm)	Magnesium (ppm)
Wellesbourne soil	6.1	32.4	37	494	140
Sand/absorbent clay	8	3.7	8	116	1859
Sid2 soil	-	-	-	-	-
Sid2 inoculant	-	-	-	-	-
Col0 soil	-	-	-	-	-
Col0 inoculant	-	-	-	-	-