Investigating the impact of soil tillage and crop rotation on the bacterial microbiome associated with winter oilseed rape under Irish agronomic conditions

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Abstract

Modern agriculture is challenged with sustaining a high quality food flow to an increasing global population. Therefore, the agriculture intensification is largely achieved through the use of farm equipment, intensive soil tillage, fertilizers, pesticides and other manufactured inputs. As a result, the farming industry is facing a significant profitability problem due to continuing high input costs, and consequently research into low input, more efficient and eco-friendly crop production systems are required. One way to assist in achieving these goals is to integrate beneficial plant microbiomes i.e., those enhancing plant growth, nutrient use efficiency, abiotic stress tolerance, and disease resistance into agricultural production. However, this plant microbiome is influenced by the agricultural management practices such as soil tillage and crop rotation which have major effect on structure, composition and function of soil, rhizosphere and endophytic bacterial microbiota.

Oilseed rape (*Brassica napus* L.) is an important break crop in cereal crop rotation and can significantly reduce the rate of fungal disease incidence and as a result, improves the yield of subsequent cereal crops. Additionally, oilseed rape is the world's third largest source of vegetable oil used in human nutrition and as a source of oil for biodiesel production. Therefore, the promotion of agricultural practices that maintain the natural diversity of the oilseed rape microbiome is receiving attention as an important element for a sustainable agricultural system that ensures crop productivity and quality, while reducing inputs. The plant microbiome can be considered as an extension of the host genome or even as the plant's second genome. Therefore, even small changes in the host due to agronomic factors may influence the plant microbiome, and these changes may even feedback to modulate the behaviour of the host.

The main objective of the current work was to explore the impact of soil tillage (plough based conventional tillage *vs* conservation strip tillage) in field-grown winter oilseed rape bacterial microbiome over the plant growth stages. We also focussed on the microbiome niche differentiation between the different plant environments (rhizosphere, root and shoot) due to influence of soil tillage. In this study a high-resolution methodology based on illumina sequencing of the bacterial 16S rRNA marker gene was adopted to characterize and compare soil and plant associated bacterial communities. Our results show that oilseed rape is preferentially colonized by Proteobacteria,

Actinobacteria, Bacteroidetes and Chloroflexi, and each bacterial phylum is represented by a dominating class or family of bacteria. In general, soil tillage was found to have a profound effect on the bacterial community structure in the endosphere of oilseed rape (especially root and shoot), without perceptible effects on the rhizospheric bacterial communities. Moreover, oilseed rape plant selected a subset of microbes at different stages of development, presumably for specific functions. Furthermore, within the bacterial community structures, we observed strong clustering according to plant compartment whereby each compartment rendered microbiota significantly dissimilar from each other where soil tillage fine-tunes their composition.

We also studied the impact of oilseed rape-wheat crop rotation, in combination with the soil tillage, to explore its influence on anti-microbial compound 2,4-diacetylpholroglucinol (2,4-DAPG) producing *Pseudomonas* spp. abundance, in rhizosphere and root of oilseed rape and wheat crops over the plant growth stages, by using the quantitative PCR technique. Overall, this study showed that crop rotation in combination with conservation strip tillage increase the population of the 2,4-DAPG^{+} *Pseudomonas* spp. in rhizosphere and root of oilseed rape and wheat crops.

Overall, this practical study has broaden an understanding of how oilseed rape and its microbiome responded to different agriculture management practices, and provided new insights into the complex relationship between oilseed rape and its associated microbes.

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List of Abbreviations

OSR	Oilseed rape	°C	Degrees Celsius
WOSR	Winter oilseed rape	%	Percent
CT	Conventional tillage	RPM	Revolutions Per Minute
ST	Strip tillage	SD	Standard Deviation
Rt	Root	SE	Standard Error
Sh	Shoot	ANOVA	Analysis of Variance
B/Bk	Bulk soil	ANCOM	Analysis of composition of microbiomes
Rz	Rhizosphere	CFU	Colony forming unit
V	Vegetative stage	OD	Optical Density
F	Flowering stage	OTU	Operational taxonomic unit
H	Harvesting stage	NGS	Next generation Sequencing
mg	Milligram	PCoA	Principle Co-ordinate Analysis
µg	Microgram	sec	Second
ng	Nanogram	H	Hour
µL	Microliter	gfp	Green Fluorescent Protein
µM	Micromolar	PCR	Polymerase Chain Reaction
ACC	1-Aminocyclopropane-1-carboxylic Acid	qPCR	Quantitative Polymerase Chain Reaction
AHL	N-acyl homoserine Lactone	NH_3	Ammonia
bp	Base Pair	NO^{-2}	Nitrite
DAPG	2,4-diacetylphloroglucinol	NO^{-3}	Nitrate
ddH_2O	Double Deionised Water	N_2	Nitrogen
dH_2O	Deionised Water	ha	Hectare
DNA	Deoxyribonucleic Acid	CN	Genome Copy Numbers
dsDNA	Double Stranded DNA	Cq	Quantitation Cycle
rDNA	Ribosomal DNA	PGPB	Plant Growth Promoting Bacteria
rRNA	Ribosomal ribonucleic acid	spp.	Species
QS	Quorum sensing	PGP	Plant growth promotion
RDP	Ribosomal Database Project	CWDE	Cell wall degrading enzyme
BC	Bray Curtis	WUF	Weighted UniFrac

Publications Associated with this Research Work

Papers

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Abstracts

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Chapter 1

General Introduction

Sections of this chapter are submitted as:

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1.1 **Introduction**

In the 21st century, modern agriculture is facing the significant challenge of producing high quality nutritious food for a continuously increasing global population, while also conserving the natural resources that are so critical to its ability to maintain productivity (Godfray *et al.*, 2010, Tilman *et al.*, 2011). In general, food crops are the most important aspect of the food system, which directly provide edible produce for human consumption, fodder for livestock (that ultimately support the meat and dairy industries, the drinks industry) and even biofuel production (Steinfeld *et al.*, 1998, Trostle, 2008). To accomplish these increasing food demands, intensive agricultural practices, excessive use of agrochemicals and heavy machinery are often employed, which can in turn have negative impacts, both on soil quality and the wider environment. For example, poor agronomic practices damage the soil physicochemical structure that can lead to soil degradation, erosion and reduce soil fertility that further induces a loss of biodiversity, and increases greenhouse gas (GHG) emissions such as CO_2, N_2O and CH_4 (Tilman *et al.*, 2002). Such emission of GHG significantly contributes to a larger global problem of climate change. According to estimates of the IPCC (2014), CH_4 and N_2O from agricultural sources account for 50 % and 60 % of total emissions, respectively. Therefore, major international research programs such as the EU Horizon 2020 programme aims to advance crop productivity by preserving the sustainability of agriculture. Soil microorganisms are the key driver of carbon-nitrogen cycling and mediate all processes leading to the production or consumption of the GHG in soils (Jena *et al.*, 2013). Therefore, it is essential to improve suitable soil management and agronomic practices for the crop cultivation that allow the manipulating of the soil microbes in a positive aspect. Moreover, the advancements in modern agriculture are multifaceted, and include the use of beneficial soil and plant microbes which have been shown to be associated with significant yield increases, while maintaining the sustainability of agro-ecosystem (Bottini *et al.*, 2004). Therefore the beneficial plant-microbe interactions are now hot-topics of research, as these have been reported to hold one of the key solutions to the challenges constraining the productivity and sustainability of agriculture (Farrar *et al.*, 2014, George *et al.*, 2016).

1.2 **Importance of the plant microbiome**

Plants develop close interactions with microorganisms that are essential for their performance and survival. Thus, eukaryotes and prokaryotes in nature can be considered as meta-organisms or holobionts. Consequently, microorganisms that colonize different plant compartments contain the plant's second genome. In this respect, many studies in the last decades have shown that plant-microbe interactions are not only crucial for better understanding plant growth and health, but also for sustainable crop production in a changing world. Plants exist in close association with the microbes which thrive and interact outside, on and inside various plant tissues designated as the plant microbiome/microbiota (Schlaeppi & Bulgarelli, 2015) that are acknowledged as key drivers for plant health, productivity, and agricultural ecosystem functioning. Establishment the relationship between the host-plant and microbiota is arbitrated through the action of compounds produced by the bacteria and host plant (Reinhold-Hurek & Hurek, 2011, Brader *et al.*, 2014). Many research studies have documented the plant growth promoting (PGP) effects on plant growth and health such as establishment of greater root systems, larger propagation output, better nutrient acquisition and uptake, enhance stress tolerance, and protection against pathogenic invasions (Whipps, 2001, Compant *et al.*, 2005, Ryan *et al.*, 2008, Glick, 2012) (Figure 1.1).

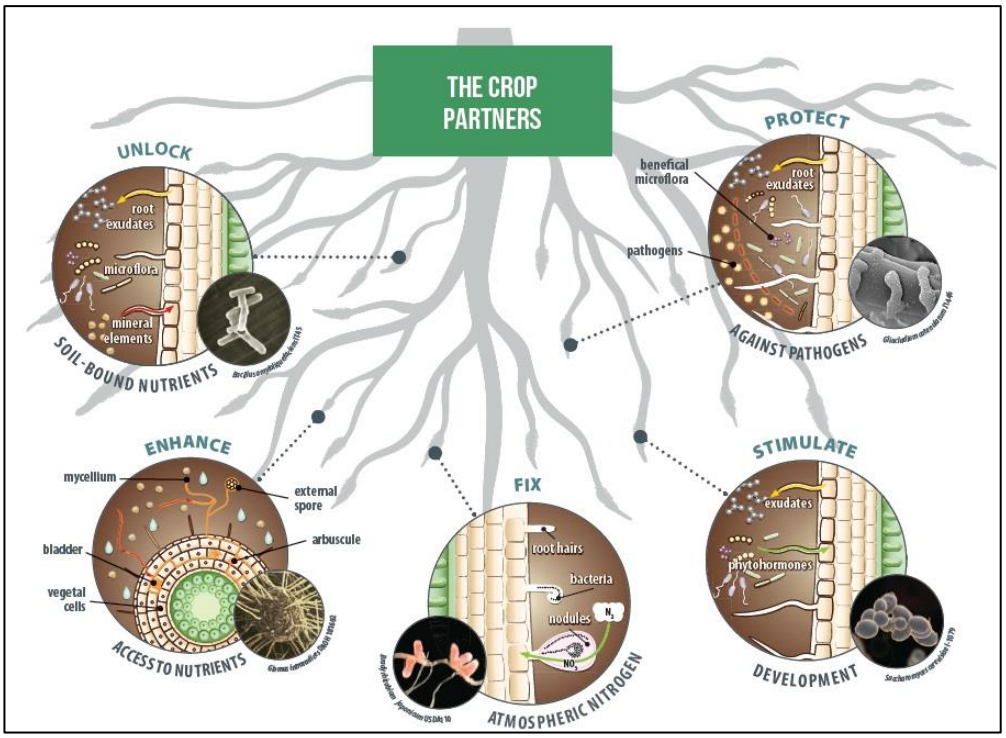

Figure 1.1 The plant microbe interactions that have been identified in rhizosphere and root microhabitats. The microbial actors in the root zone work in concert to stimulate nourish and protect the plants. (Adapted from http://www.lallemandplantcare.com)

1.2.1 Plant nutrient acquisition

Nitrogen is essential to all living organisms as it is a vital component of nucleic and amino acids. N_2 gas makes up 78 % of the atmospheric volume. The necessity of nitrogen for cell function and structure makes it one of the central and most important biochemical elements. N_2 cannot be used by all organisms and so needs to be changed to an utilisable form. This process is called nitrogen fixation, where gaseous dinitrogen is transformed into ammonia by the nitrogenase enzyme complex ($N_2 + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$). Bacteria capable of facilitating this reaction are referred to as nitrogen fixing bacteria (Bhattacharjee *et al.*, 2008). These microorganisms can provide nitrogen for plants and consequently benefits to the plant growth. Free-living nitrogen fixing bacteria convert atmospheric nitrogen (N_2) to ammonia (NH_3) using the energy from ATP through the ammonification process. Nitrifying bacteria then convert this ammonia to nitrite (NO_2^-) and then to the plant available form, nitrate (NO_3^-) though the nitrification process. Under oxygen limiting

conditions in soil, the denitrifying bacteria convert nitrate to free atmospheric nitrogen. In this way bacteria play vital roles in maintaining the nitrogen cycle. Legume plants have developed a symbiotic relationship with some nitrogen fixing bacteria, and they form nodules as a part of their root structure. These nodules contain endophytes that are capable of fixing nitrogen (Hirsch, 1992). In most agricultural fields, nitrogen fertiliser has been applied to crops in the plant available form of nitrate to reduce the deficiency of nitrogen in soil as well as to boost and maintain crop yields. The applications of nitrogen, however, have detrimental effects to the environment as its application produce greenhouse gases, it pollutes ground waters and causes blooms of primary producers in local waterways (Tilman *et al.*, 2002, Tilman *et al.*, 2011). Through more extensive use of plant growth promoting/nitrogen fixing bacteria in agriculture, the use of such chemical fertilisers can be reduced. For instance, significant nitrogen fixation under field conditions has been shown in sugarcane and rice, mostly using the ^{15}N natural abundance technique, with Brazilian sugarcane varieties having at least 40 kg fixed N hectare^{-1} year^{-1} (Urquiaga *et al.*, 2012). Therefore, utilising nitrogen fixation associations between bacteria and plants have potential environmental benefits as this technology could significantly reduce the need for chemical nitrogen applications.

Phosphorus is considered the second most important nutrient for plant growth, it plays a structural role in RNA, DNA and phospholipid formation, it transports energy in the form of adenosine triphosphate (ATP) and therefore it is a key element to a plant's growth and development. Strategies to improve phosphorus availability/uptake can contribute significantly to plant growth, because less than 5 % of the phosphorus content of soils is bioavailable to plants. Microorganisms with the capacity to solubilize mineral phosphorus are abundant in most soils (up to 40 % of the culturable population). Well-known isolates belong to *Bacillus*, *Pseudomonas*, or *Penicillium* genera. Mineralization/solubilisation is achieved by the production of organic acids (such as acetate, succinate, citrate, and gluconate) or phosphatases, liberating orthophosphate from inorganic and organic phosphorus pools. Several genes involved in phosphorus solubilisation have been found and characterized (Rodríguez *et al.*, 2007). Organic matter such as dead animal and plant tissues including sloughed off cells in the rhizosphere contain bound forms of phosphorous that also are inaccessible until released though decomposition. Bacteria produce single step enzymes that release insoluble phosphorus. Gluconic acid and 2-ketogutarate as a by-product of glucose metabolism

appears to be one of the main mechanisms of phosphate mineralisation (Miller *et al.*, 2010). Glucose present in the environment is oxidised as a result of a glucose dehydrogenase reaction coded for by *gad* (for gluconic acid production) and a further reaction converting gluconic acid to 2-ketogluterate with gluconate dehydrogenase coded for by the *gdh* gene. These organic acids build up on bacterial cell walls and diffuse into the environment causing a lowering of the pH, this provides metal complex anions and protons helping induce phosphorus mineralisation from insoluble phosphate compounds (Rodríguez & Fraga, 1999, Chen *et al.*, 2006, Miller *et al.*, 2010). The activity of phytase produced by microbes can also free phosphorus from soil phytate (Martínez-Viveros *et al.*, 2010).

1.2.2 Modulation of Phytohormone levels

Phytohormones are chemical compounds that promote and influence plant growth and development. Phytohormones are often divided into five major classes; auxins, cytokinins, gibberellins, abscisic acid, and ethylene although other compounds with hormonal activity have also been identified, such as strigolactones and brassinosteroids (Santner & Estelle, 2009). Microbes are enabled with genetic machinery to produce such compounds and promote plant growth in natural environments. Also, phytohormonal production of single or multiple compounds by soil- and plant-associated bacteria are frequently observed in the growth medium in laboratory conditions (Persello-Cartieaux *et al.*, 2003). However, the extent to which these contribute to plant growth promotion requires further investigations to include all compounds produced by these microbes. Therefore, this section only documents the auxin production and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity by the microbes as plant beneficial compounds.

The family of molecules with auxin activity is involved in many aspects of plant growth and development. The most abundant member is indole-3-acetic acid (IAA) and the main precursor for biosynthesis of IAA is tryptophan. Auxin production is a common feature of many soil- and plant-associated bacteria (Idris *et al.*, 2007). Multiple biosynthesis pathways have been found active in plant-associated microorganisms. The best-characterized auxin biosynthesis routes in bacteria are designated to indole-3-acetamide (IAM) and indole-3-pyruvate (IPyA) pathways (Mashiguchi *et al.*, 2011).

The observation that many PGP bacteria could produce IAA, in combination with inoculation experiments with mutant strains altered for auxin production, has led to the conclusion that auxin production is a major plant growth promoting trait (Spaepen *et al.*, 2007). For, *Azospirillum brasilense*, a direct link between IAA production and altered root morphology was demonstrated in wheat inoculation experiments where a mutant strain defective in IPyA decarboxylase could not induce the same morphological changes (Dobbelaere *et al.*, 1999). In greenhouse experiments with wheat under sub-optimal nitrogen fertilization, plants inoculated with the wild-type strain had a higher yield than control plants or plants inoculated with the mutant strain (Spaepen *et al.*, 2008). It was hypothesized that bacterial auxin production leads to root proliferation and a higher total root surface area, which in-turn allows the plant to absorb more nutrients and water from the soil (Lambrecht *et al.*, 2000).

The phytohormone ethylene was first described as a fruit-ripening hormone, but is now known to have a much broader role in other processes including senescence, abscission, and pathogen-defence signalling. Under diverse stresses, ethylene biosynthesis is induced, thereby inhibiting root and plant growth (Abeles *et al.*, 1992). Some microorganisms can interfere with ethylene biosynthesis by expression of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, encoded by the *acdS* gene. This enzyme converts the ethylene precursor ACC to α-ketobutyrate and ammonia. These microorganisms can enhance plant growth by metabolizing ACC exuded by plant roots. As the ACC concentration outside the roots decreases, ACC exudation increases and ethylene biosynthesis decreases inside the plant cells owing to the lack of precursor. This attenuates ethylene-dependent inhibitory responses and therefore increases plant growth, especially under stress conditions (Glick *et al.*, 1998, Glick *et al.*, 2007). The importance of ACC deaminase activity in plant growth promotion has been extensively studied not only by using mutants but also by overexpressing *acdS* in plants. *acdS* expressing microorganisms and plants are able to alleviate the growth inhibition induced by ethylene synthesis under stress conditions, such as flooding, drought, toxic compounds, and pathogen attack (Glick *et al.*, 2007).

1.2.3 Biocontrol against plant pathogens

Biological control or biocontrol is the process of suppressing deleterious/pathogenic living organisms by using other living organisms and ultimately contributing to plant protection. Bacteria provide many biocontrol inducing traits that include production of antimicrobial agents, competition for nutrients, production of lytic enzymes and the degradation and alleviation of various virulence factors.

Microorganisms can synthesize a wide range of compounds with antimicrobial activity. These compounds can be derived from the secondary metabolism or are (modified) proteinaceous molecules derived from ribosomal synthesis or non-ribosomal peptide synthesis. The production of antimicrobial compounds has been extensively studied in *Pseudomonads, Bacilli,* and *Trichoderma* species, including the identification of biosynthesis pathways and their regulation (Berg, 2009). Most commercial biocontrol products contain strains belonging to these groups. Well-known and characterized compounds are phenazines, 2,4-di-acetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, cyclic lipopeptide surfactants, zwittermycin A, and bacteriocins (Emmert & Handelsman, 1999, Weller, 2007, Berg, 2009, Perez-Garcia *et al.*, 2011). As for most antimicrobial compounds, biosynthesis is regulated by two component- regulatory systems and environmental conditions (Dolan *et al.*, 1993). Although biocontrol strains do not directly promote plant growth, they can influence PGP bacteria that directly stimulate plant growth, as illustrated for a 2,4-Diacetylphloroglucinol (2,4-DAPG) producing *P. fluorescens* strain that enhances the phyto-stimulatory effect of *A. brasilense* by altering the expression of genes involved in plant growth promotion. The authors of this study speculated that 2,4-DAPG is a signal molecule that coevolved in complex plant-microbe interactions (Combes-Meynet *et al.*, 2011).

Iron is abundant in soil and an essential nutrient in all life forms, but it is not very available to plants owing to the low solubility of Fe^{3+} oxides. Bacteria can produce compounds that bind to ferric iron helping the transportation of iron across cell membranes. Microorganisms can release organic acids and a broad range of siderophores to bind iron usually under iron-limiting conditions (Crosa & Walsh, 2002). In this way, a complex competition for iron occurs in the rhizosphere between different microorganisms. Therefore, siderophore production has been associated with biocontrol for this reason (Kloepper *et al.*, 1980, Beneduzi *et al.*, 2012). Moreover, PGP bacterial siderophores generally have a higher affinity for iron whereas pathogens producing

siderophores have low iron affinity or they don't produce them at all. Evidence suggests that siderophore producing strains can contribute to the availability of iron to plants and so it's not only involved in biocontrol but also encompass plant stress regulation and nutrition (Bar-Ness *et al.*, 1992). For instance, most soil-derived fluorescent pseudomonads can efficiently scavenge iron via siderophore production (e.g., pyoverdine). In this way, they antagonize some fungal plant pathogens (e.g., *Fusarium oxysporum*) and restrict their growth in the rhizosphere, thereby enhancing plant health indirectly (Benhamou *et al.*, 2002).

Hydrolases are lytic enzymes that can reduce the impact of pathogens when produced by bacterial strains, due to their ability to degrade cell wall structures. These hydrolytic enzymes include chitanase, β1,3-glucanase and proteases (Kumar *et al.*, 2012, Hamid *et al.*, 2013). Filamentous fungal cell wall is composed of chitin, glucans, polysaccharides and proteins. These aforementioned lytic enzymes can degrade fungal cell walls and have been shown to disrupt pathogenic fungal growth *in vitro* and inhibit spore germination and germ tube formation (Viterbo *et al.*, 2002).

PGP bacteria can inhibit the invasive and disease inducing effects of some pathogens by metabolising or suppressing their toxins or virulence factors. This includes the production of proteins that bind to pathogenic toxins, for example resistance protein that binds to the toxin albicidin. Other mechanisms include the hydrolysation of toxins such as fusic acid produced by *Fusarium* fungi or the degradation of albicidin (Maheshwari, 2013). Quorum sensing (QS) is a signalling process in which bacteria communicate to contribute to population densities on surfaces. Bacteria produce N-acyl homoserine lactone (AHL) and can determine the density of their communities by secreting AHL, its concentration in the population indicates the growth level of bacteria. This mechanism then triggers population responses. The QS signal regulates certain genes involved in virulence such as cell wall degrading enzymes.

1.3 **Recruitment of the plant microbiota**

The ecological studies indicate that the rhizosphere community structures of field grown plants can contain diverse group of bacteria with the endophytic component composed of a subset of this rhizosphere/rhizoplane community or with the obligate endophytes

potentially derived from a common seed endophytic community. The establishment of microbes through niche differentiation between the plant environments are the important aspect of plant-microbiota research. Soil and virtually all plant tissues host bacterial communities; at the soil (soil interface; starting inoculum of the plant microbiota), soil-plant interface (rhizosphere environment), inside the plant tissues (endosphere environment harbouring the endophytes) and at the air-plant interface (phyllosphere environment). To a lesser extent, bacterial colonization can also be distinguished in the anthosphere (flower), the spermosphere (seeds), and the carposphere (fruit). All these microenvironments provide specific biotic and abiotic conditions for the residing bacterial communities. Here we will focus on the soil, rhizosphere, endosphere and phyllosphere environments for bacterial plant-microbiota establishments.

Bulgarelli *et al.* (2013) inferred some general principles concerning niche differentiation from the available literature. In the bulk soil biomes, edaphic factors determine the structure of the bacterial communities where after the first differentiation step, rhizodeposits (and the resulting chemo-tactic effects) and host cell wall features of the plant roots fuels a substrate-driven community shift to form distinctive rhizosphere microbiomes. Subsequently, host genotype-dependent selection, with the plant innate immune system as main driving factor, of communities thriving on the rhizoplane leads to differentiation of the endophytic microbiota within the plant roots. Further, niche differentiation in the aerial plant compartments (stem and leaf endosphere) was suggested by Bulgarelli *et al.* (2013), but is yet to be validated by experimental data. Furthermore, the bacterial colonization is also determined by the specific traits for instance, expression of genes involved in chemotaxis, the formation of flagella and pilli, and the production of cell-wall degrading enzymes such as cellulases, pectinases which helps in ascending migration of bacteria in plant (Capdevila *et al.*, 2004, Compant *et al.*, 2010, Bulgarelli *et al.*, 2012). Chi *et al.* (2005) demonstrated the endophytic bacterial colonization and their movement from rhizosphere/rhizoplane followed by colonization within roots, and then ascending migration into the stem base, leaf sheath and leaves in rice plant using the *gfp*-tagged rhizobia, and stated that root is the main entry point of potential endophytes from soil to host plant. The schematic representation of the recruitment of endophytic microbiome is displayed in Figure 1.2.

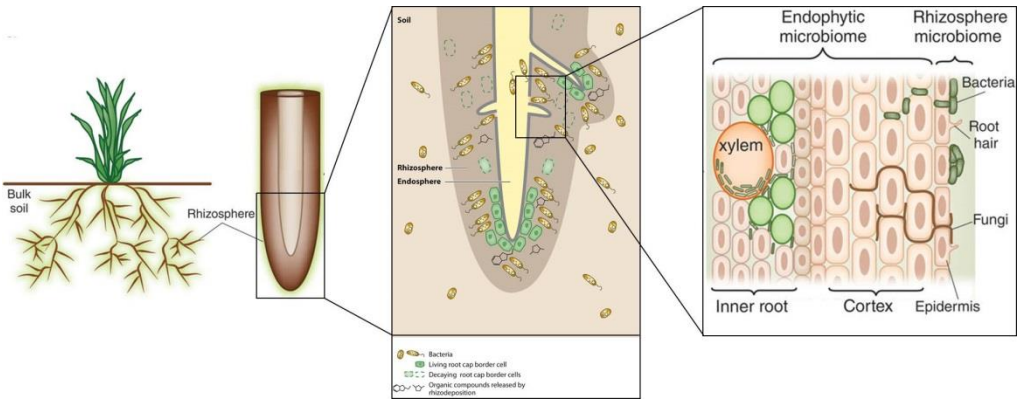

Figure 1.2 Microbiome niche differentiation at the root-soil interface. From outside to inside, the habitats are the bulk soil, rhizosphere, and endosphere. Rhizodeposits generated from root cap border cells and the rhizodermis provoke a shift in the soil biome. Cellular disjunction of the root surface during lateral root emergence provides a potential entry gate for the rhizosphere microbiota into the root interior. The rhizosphere microbiome includes bacteria and fungi that are recruited from bulk soil and colonize the root surface. The endophytic microbiome includes species that infiltrate the root cortex and live as endophytes until release back into the soil upon root senescence. (Adapted from Bulgarelli *et al*., 2013, Hirsch & Mauchline, 2012)**.**

1.3.1 Soil bacterial microbiota

Soil is the most diverse and intricate biomaterial on the earth (Young & Crawford, 2004). For instance, a single gram of soil can comprise more than 1 billion bacteria and more than 10,000 species per gram. These facts provide evidence that soil biodiversity is enormously high (Roesch *et al.*, 2007). Moreover, soil comprises remarkably higher quantity and diversity of microorganisms compared to other environments, even though microbes would only occupy 10^{-6} % of the soil surface area (Young & Crawford, 2004). This soil biodiversity can be explained by the existence of a multitude of microhabitats with variations in physicochemical properties, soil structure, energy sources and carbon sources (Alegria Terrazas *et al.*, 2016). The bacterial communities flourishing in association with roots are mainly recruited from the surrounding soil biome and modulated by the plant species (Bulgarelli *et al.*, 2013). For example, a detailed characterization of the microbiome of *Arabidopsis thaliana* through high-

throughput sequencing revealed that different soil types are the driver of root-associated bacterial community structure and composition (Bulgarelli *et al.*, 2012, Lundberg *et al.*, 2012). However, the same study failed to detect root microbiota where plants were grown under axenic condition with surface sterilized seeds (Bulgarelli *et al.*, 2012, Lundberg *et al.*, 2012). Likewise, rice seedling germinated under axenic conditions appear sterile, however after transplantation in soil, seedlings commenced to assemble distinct microbiota within a few days (Edwards *et al.*, 2015). These observations indicated that a limited number of bacterial taxa may be transmitted though seed, and that soil may be the origin of microbiota at least partially, for above ground plant organs. Moreover, soil microbial community structure and the associated environmental parameters are also more important drivers of root associated bacterial community than plant genotypes and species as demonstrated in various studies (Bulgarelli *et al.*, 2012, Lundberg *et al.*, 2012, Schlaeppi *et al.*, 2014). Taken together these observations demonstrate that soil biota is the starting inoculum for the microbial communities that thrive in association with plants.

1.3.2 Rhizosphere bacterial microbiota

More than a century ago, Lorenz Hiltner defined the rhizosphere as the interface of soil and roots, which is influenced by the release of root exudates (Hartmann *et al.*, 2008). Due to the importance of the soil habitat of plants, a significant amount of research now focuses on the rhizosphere environment (Bakker *et al.*, 2012, Berendsen *et al.*, 2012, Mendes *et al.*, 2013). The rhizosphere is a hot spot for numerous organisms and is considered as one of the most complex ecosystems on Earth (Hinsinger *et al.*, 2009). Organisms found in the rhizosphere include bacteria, fungi, oomycetes, nematodes, protozoa, algae, viruses, archaea, and arthropods, which recently had led to the emergence of the term ‘rhizosphere zoo’ (Raaijmakers *et al.*, 2009, Turner *et al.*, 2013). Specifically, the rhizosphere microbiome is of central importance not only for plant nutrition and health but also contributes substantially to microorganism-driven carbon sequestration, which has an important role in ecosystem functioning and nutrient cycling in terrestrial ecosystems (Philippot *et al.*, 2009, Mendes *et al.*, 2013, Berg *et al.*, 2014). In contrast to non-rooted bulk soil, the rhizosphere is characterized by much

higher bacterial abundances and activities, collectively termed "the rhizosphere effect" (Walker *et al.*, 2003, Hartmann *et al.*, 2008).

The major driving force in the regulation of the microbial diversity and activity in the rhizosphere soil and rhizoplane (external root surface) and ultimately the formation of distinctive rhizosphere microbiota from soil biomes is the deposition of large amounts of organic carbon by the plant roots in a process termed rhizodeposition (Bertin *et al.*, 2003, Dennis *et al.*, 2010). Rhizodeposition comprises the release of (1) soluble root exudates by root dermal cells, (2) insoluble mucilage by the root cap, (3) root cap and border cells and (4) carbon to root-associated symbionts, death and lysis of root cells. Soluble root exudates contain a variety of compounds, predominantly organic acids and sugars but also inorganic acids, amino acids, phytosiderophores, fatty acids, vitamins, growth factors, hormones, purines, nucleosides and antimicrobial compounds (Jones *et al.*, 2009). Together these root exudates are key determinants of rhizosphere microbiome structure (Shi *et al.*, 2011, Badri *et al.*, 2013). The composition of the plant root exudates is highly variable between plant species, cultivars and moreover with plant age and developmental stage resulting in specific bacterial communities (Mark *et al.*, 2005, Cavaglieri *et al.*, 2009, DeAngelis *et al.*, 2009, Inceoglu *et al.*, 2010, Chaparro *et al.*, 2013, Chaparro *et al.*, 2014). It now appears that in addition to carbohydrates and amino acids, which act as general chemical determinants in the rhizosphere, secondary metabolites such as plant-specific flavonoids also play a role in the development of plant-specific microbial communities in the rhizosphere (Badri *et al.*, 2013).

Most authors consider the process of rhizo-deposition as active plant-microbe signalling, at significant carbon cost, whereby plants may modulate the rhizosphere microbiome to their benefit by selectively stimulating microorganisms with traits that are beneficial to plant growth and health. However, others have argued that exudates are passively 'released' as overflow/ waste products of the plant (Jones *et al.*, 2009, Dennis *et al.*, 2010). Since rhizo-deposition accounts for around 11 % of the net photosynthetically fixed carbon and 10-16 % of total plant nitrogen, varying greatly depending on plant species and plant age (Jones *et al.*, 2009, Mendes *et al.*, 2011), defining the process as waste products seems highly implausible. However, root exudation is not a unidirectional flux and plant roots can take up a range of exuded compounds from the rhizosphere into the roots and transfer them again to shoots. In any

case, soil microorganisms are chemotactically attracted to the plant root rhizodeposits, after which rhizosphere/rhizoplane competent bacteria proliferate in this carbon rich environment and form distinctive rhizosphere communities (Lugtenberg & Kamilova, 2009).

1.3.3 Endosphere bacterial microbiota

In addition to bulk soil and rhizosphere communities, large and diverse bacterial populations live inside plants without causing detrimental effects or cellular damage to the plant. These communities of microbes are collectively termed endophytes. Bacterial endophytes have been isolated from virtually all plant species studied. Many endophytic inhabitants of plants are now often recognized as having unique, intimate and crucial interactions with the plant (Hardoim *et al.*, 2008, Ryan *et al.*, 2008, Hirsch & Mauchline, 2012, Bulgarelli *et al.*, 2013, Turner *et al.*, 2013). The majority of endophytes are widely considered as being a sub-population of the rhizosphere microbiome, since their primary colonization route are through the plant roots (Hardoim *et al.*, 2008, Compant *et al.*, 2010, Turner *et al.*, 2013). However, endophytes also display characteristics distinct from rhizospheric bacteria suggesting that not all rhizospheric bacteria can enter plants and/or that after colonization of their host plant, endophytes have the potential to modulate their metabolism and become adapted to their internal environment (Ferrara *et al.*, 2012, Monteiro *et al.*, 2012, Sessitsch *et al.*, 2012). In order to transition from the soil to the plant, free-living soil-borne bacteria must first demonstrate rhizosphere and/or rhizoplane (external root surface) competence, i.e. having the ability to colonize the rhizosphere and/or rhizoplane during an extended period in an environment characterized by strong microbial competition where after establishment in the host plant can follow (Compant *et al.*, 2010).

Rhizosphere/rhizoplane competence is predominantly determined by the ability of soil bacteria to approach plant roots via chemotaxis-induced motility to plant root rhizodeposits (Jones *et al.*, 2009, Compant *et al.*, 2010). In addition to chemotaxis towards exudates, mucilage and other rhizodeposits, characteristics such as bacterial flagella, quorum sensing as well as the production of specific compounds/enzymes are involved in the rhizosphere/rhizoplane colonization process (Turnbull *et al.*, 2001, Bohm *et al.*, 2007, Cho *et al.*, 2007). Furthermore since root exudates and mucilage derived nutrients attract a myriad of organisms to the rhizosphere environment,

beneficial plant-associated bacteria have to be highly competitive to successfully colonize the root zone (Raaijmakers *et al.*, 2009, Mendes *et al.*, 2013). The production of secondary metabolites involved in biocontrol (such as siderophores, lytic enzymes and antibiotics) provide some bacteria a selective and competitive advantage against other microorganisms, thereby contributing to their rhizocompetence (Lugtenberg & Kamilova, 2009, Raaijmakers *et al.*, 2009). After rhizoplane colonization, endophytic competence, i.e. the ability to successfully colonize the host plant involves several specific characteristics (Compant *et al.*, 2010). Most evidence suggests that endophytic bacteria enter their host plant at naturally occurring cracks in the roots and/or root tips such as those found at root emergence sites (lateral root junctions) or those created by deleterious microorganisms (James & Olivares, 1998, James *et al.*, 2002, Monteiro *et al.*, 2012).

The colonization process does not necessarily involve active mechanisms and thus all rhizosphere bacteria can be expected to be endophytic at one stage of their life (Hardoim *et al.*, 2008). Especially the progression from the rhizoplane to the root cortex may occur via passive mechanisms at natural breaks in root and/or root tips. However, further endophytic colonization, crossing barriers such as the endodermis and pericycle in the root cortex, is unlikely to be an entirely passive process (Compant *et al.*, 2010). Although the endodermal cell layer is often disrupted by the formation of secondary roots (which derive from the pericycle) (Casimiro *et al.*, 2003) or the action of deleterious bacteria, many endophytic bacteria express cell-wall-degrading enzymes (CWDEs) such as cellulases and polygalactorunases which actively dissolve the complex polysaccharides that form the middle lamella between adjacent plant cells. However these enzymes are generally produced in much lower concentrations than expressed by plant pathogens (Monteiro *et al.*, 2012) but provides just enough disruption to allow the bacteria to pass between the cells. After passing through the endodermis barrier, endophytic bacteria have to penetrate the pericycle to further reach the root xylem vessels of their hosts and once they have made it here they can then spread systematically inside the plant colonizing the stems and leaves (Hardoim *et al.*, 2008, Compant *et al.*, 2010). Endophyte numbers are generally lower in aerial parts than in roots, which suggests that although there is some upward movement of endophytes within their hosts, supported through the plant transpiration stream, this movement is limited, and may only be possible for bacteria that express CWDEs and/or Type-3-

secretion systems (Compant *et al.*, 2010, Monteiro *et al.*, 2012). In addition, amongst others lipopolysaccharides, flagella, pili, and twitching motility have been shown to affect endophytic colonization and bacterial mobility within host plants (Dorr *et al.*, 1998, Bohm *et al.*, 2007).

1.3.4 Phyllosphere bacterial microbiota

In addition to the studies on plant microbiota associated with below ground plant organs, numerous research studies evidence the substantial abundance and diversity of microbial denizens residing either inside or outside the aerial tissues of plants (Peñuelas & Terradas, 2014, Bulgarelli *et al.*, 2015). The aerial outer surface of a plant with the bulk of the surface provided by the green leaves and stems are known as the phyllosphere. The phyllosphere embodies an environment much more dynamic than the rhizosphere where buffered fluctuations of abiotic conditions prevail. Resident microbes are subjected to large fluctuations in temperature, moisture and UV light radiation throughout the day/night and moreover have limited access to nutrients (Hirano & Upper, 2000). Unlike root exudation, which releases significant amounts of photosynthetically fixed carbon into rhizosphere, no mechanism exists for the constant supply of soluble organic compounds to leaf and/or stem surfaces.

Microbial colonization of leaves is not homogenous but is affected by leaf structures such as veins, hairs and stomata and microbial phyllosphere communities are highly variable (intraspecies and temporal) with reduced complexity (Lindow & Brandl, 2003, Redford *et al.*, 2010). A few bacterial genera, including *Pseudomonas, Sphingomonas, Methylobacterium, Bacillus, Massilia, Arthrobacter* and *Pantoea*, appear to compose the core of phyllosphere communities (Delmotte *et al.*, 2009). The low-complexity phyllosphere communities may originate from several sources: (1) air and its aerosols, which flow around the leaves and moreover abundant sequences assigned to *Sphingomonas* and *Pseudomonas* were identified in clone libraries of several aerosol samples (Fahlgren *et al.*, 2010), (2) neighbouring plants and plant debris, and (3) water, as reported by Delmotte *et al.* (2009) in a metaproteogenomics approach to compare the phyllosphere communities of paddy field grown rice (Oryza sativa) plants in relation to the flooding water of the paddy field. Proteogenomic analyses of various phyllosphere microbiomes have revealed differential adaptation

strategies to the leaf environment (Delmotte *et al.*, 2009). These analyses revealed the species that assimilate plant-derived ammonium, amino acids and simple carbohydrates, implicating these compounds as primary nitrogen and carbon sources in the phyllosphere. These studies also observed that *Methylobacterium spp*., a widely abundant phyllosphere microbe, expresses proteins for the active assimilation and metabolism of methanol, a by-product of plant cell wall metabolism derived from pectin. These bacteria seem to adapt to the phyllosphere via a specific methylotrophic one-carbon metabolism. Reversely, *Sphingomonas spp*. contained multiple transport proteins (e.g. TonB-dependent receptors) indicating a large substrate spectrum as an adaptation strategy to low-nutrient availability.

1.4 **Determining the composition of plant microbiome**

There are two major approaches that can be used to investigate the bacterial microbiota; cultivation-dependent and -independent characterization. For decades, microbial research relied on cultivation based methods to characterise plant-associated bacterial communities (Hardoim *et al.*, 2008, Compant *et al.*, 2010, Turner *et al.*, 2013). Culture-dependent studies provide (a) detailed information about specific, readily isolated bacteria, (b) isolated pure bacterial cultures suited for genome sequencing and the unravelling of genes responsible for efficient rhizospheric and endophytic colonization and/or genes responsible for plant-growth promotion mechanisms (Krause *et al.*, 2006, Redondo-Nieto *et al.*, 2012) and (c) collections of phenotypically screened bacterial strains (e.g. plant growth promotion characteristics, metal resistance, potential to degrade organic pollutants), which can be exploited for example to improve biomass production and/or phytoremediation (Germaine *et al.*, 2009) or for the production of secondary metabolites (Brader *et al.*, 2014). In contrast to their advantages, culture-dependent studies also entail some inherent drawbacks including (i) most microbes are tremendously difficult to culture in the laboratory, (ii) give an incomplete view of microbiome and lacking the sensitivity to detect small shifts in community compositions, and (iii) introducing significant bias in the taxa they identify and drastically limiting community diversity estimates (Lebeis, 2014).

More recently, the development and implementation of massive parallel sequencing technologies (next-generation sequencing) and their corresponding

bioinformatics tools have revolutionized the methods for studying microbial ecology by enabling high-resolution community profiling. These sequencing technologies are the cornerstones of the culture-independent characterization of plant microbiota. Sequencing based bacterial microbiota studies typically fall into two categories; (1) targeted amplicon based sequencing such as 16S ribosomal RNA (rRNA) gene, a phylogenetic marker molecule to provide the detailed picture in terms of bacterial diversity and community composition, or (2) shotgun sequencing, which provide a comprehensive overview of the potential functional roles of microbes in a specific environment.

Amplicon sequencing is the most extensively used technique for describing the diversity of microbiota. Here, the microbial community is sampled (e.g. soil, plant tissues), and DNA is extracted from all the cells in the sample. The 16S rRNA is both taxonomically and phylogenetically informative marker for prokaryotes (Pace *et al.*, 1986) that is targeted and amplified by PCR. The ensuing amplicons are sequenced and analysed bioinformatically to determine the presence/absence of microbes and their relative abundance. This amplicon sequencing has revealed the tremendous amount of bacterial diversity on earth (Pace, 1997, Rappe & Giovannoni, 2003) and has been used to characterise the bacteria from various plant tissues and species (Germida *et al.*, 1998, Berg *et al.*, 2005, Bulgarelli *et al.*, 2012, Lundberg *et al.*, 2012, De Campos *et al.*, 2013, Jin *et al.*, 2014, Schlaeppi *et al.*, 2014, Edwards *et al.*, 2015, Beckers *et al.*, 2017). Comparing 16S sequence profiles across samples clarifies how microbial diversity associates with and scales across the environmental conditions, generate insights into host-microbe interactions (Bulgarelli *et al.*, 2012), and compares microbiota associated with cohort of hosts of distinct genotypes or treatment conditions (Lundberg *et al.*, 2012). However, this sequencing technique also has some limitations, for example, (a) it may fail in resolution of substantial fraction of bacterial diversity due to various biases associated to PCR (Hong *et al.*, 2009, Logares *et al.*, 2014), (b) different taxonomic assignment methods vary radically in their ability to recapture the taxonomic information in full-length 16S rRNA sequences (Liu *et al.*, 2008, Schloss, 2010), (c) only provides the information of taxonomic composition of bacterial community, (d) 16S PCR primers also amplify the plant derived plastidial and mitochondrial sequences which can contaminate and interfere with the characterization of the plant endophytic microbiota. This signifies a severe issue while deciphering the endophytic component of

the plant microbiota, which has to be bypassed by using dedicated protocols proposed by Lundberg *et al.* (2013). Additionally, while designing the targeted amplicon based sequencing experiment, the important aspect should be considered about the choice of PCR primers that influence the outcome of the analysis. Thus, it is important to rely on established protocols and/or assess the performance of different PCR primers, compare multiple platforms and approaches before starting the full-blown experiment (Walters *et al.*, 2016).

1.5 **Agronomic importance of *Brassica napus***

Brassica napus L. (oilseed rape) is the most economically important species of *Brassica* worldwide (Etesami & Alikhani, 2016). Oilseed rape (OSR) is also known as rapeseed, swede rape, argentine rape, oil rape or canola. In terms of the world market the term 'Rape' is used to describe oil seeds of various plant species. Within the genus *Brassica*, there are many combined species and many subspecies. Differences occur between the species as a result of genomic features, the chromosome numbers for *Brassica campestris, Brassica oleracea* and *Brassica nigra* are diploid (2n) whereas *Brassica napus, Brassica carinata* and *Brassica juncea* contain tetraploid (4n) sets of chromosomes. The close genetic relationship between *Brassica* species makes the crop favourable for crossing in selective breeding programs. There are two of types of *Brassica napus* crops grown for harvest viz., winter and spring OSR. Many varieties of both winter and spring OSR exist; those selected for cropping are usually picked based on the suitability of their phenotypes. Such characteristics include yield, earliness of maturity, standalone power, disease resistance and environment resistance. OSR can be further divided into three sub categories double low, HEAR (high erucic acid rape) and HO; LL (high oleic; low linolenic fatty acid profile). Double low varieties are the most commonly produced varieties in Ireland, as they are suitable for human consumption. They have a typical profile of <2 % measured fatty acids and are low in glucosinolates and erucic acid and have an oil content of 43-44 %. HEAR variants are not considered suitable for human consumption as they contain high levels of erucic acid an immunotoxin and gluconinolates fatal to animals and humans at high levels; they are grown for biodiesel production. HO;LO variants are most commonly associated with

food processing as they are best suited for human consumption (Orlovius, 2003, Teagasc, 2016).

OSR has been grown in Ireland for over 40 years. It was first grown in Ireland in 1970 but was only intensively produced since 1980. The rate of its growth has been directly associated with domestic demands. Its use as an alternative break crop is now favoured over sugar beet since the demise of the sugar industry in Ireland. OSR is in high demand due to its uses in a variety of sectors including; human nutrition as the oil has a high proportion of unsaturated fatty acids (linoleic acid 15-20 % and linolenic acid 8-12 %), a valuable animal feed with high energy and protein content, the production of renewable biodiesel fuel and environmental friendly lubricating oil (Cardone *et al.*, 2003, Delourme *et al.*, 2006). Not only that, OSR is an important break crop in cereal crop rotation and can significantly reduce the rate of 'take-all' fungal disease (*Gaeumannomyces graminis* var. *tritici*) and as a result, increases the yield of the following cereal crops by 20-30 % (Angus *et al.*, 1991, Kirkegaard *et al.*, 2008). Long-term experiments reported that OSR improves the yield of wheat grown in rotation with OSR by 10-26 % compared to wheat in monoculture or wheat in rotation with other cereal crops such as barley and oats (Schonhammer & Fischbeck, 1987, Christen *et al.*, 1992). Moreover, the deep tap root system of OSR can improve soil structure and tilth by improving soil aggregates, porosity and increasing water and nitrogen uptake which is important for healthy growth of a subsequent cereal crop (Kirkegaard *et al.*, 2008). OSR is grown as winter (WOSR) or spring (SOSR) varieties. The winter varieties are established in early autumn thus it can take up considerable quantities of nitrogen and reduce the nitrate leaching from the soil. In addition, it covers the ground due to its large biomass and reduces the risk of surface runoff, soil erosion, and soil acidification (Teagasc-report, 2009). Due to its exceptional economic and environmental impacts, OSR has become an important crop for the farming community. The total area of OSR cultivation is increasing rapidly and in 2016, the FAO reported that OSR cultivation has increased by 79 % from 1989 with over 71 million tons of rapeseed was being produced worldwide.

1.6 Agricultural management practices as drivers of bacterial microbiome selection

Agricultural land management greatly alters the physical, chemical and biological properties of soils (García-Orenes *et al.*, 2013). Soil tillage may lead to reduction in soil microbial diversity due to mechanical destruction, soil compaction, reduced pore volume, desiccation and disruption of access to food resources (Giller, 1996). The excessive use of pesticides and fertilizers can drastically modify the function and structure of microbial communities, thereby altering the normal functioning of terrestrial ecosystems (Pampulha & Oliveira, 2006). Agricultural management alters the quantity and quality of plant residues entering the soil and their spatial distribution, through changes in nutrients and inputs. To improve or maintain the soil quality, biodiversity and reduce soil erosion, the development and implementation of new sustainable agricultural practices an integrated approach must be encouraged. For example, conservation no-tillage or reduced tillage, crop rotation, use of plant growth promoting bacteria (PGPB) inoculants, mineral/organic fertilisers, disease and drought resistant varieties (García-Orenes *et al.*, 2010). It is very important to understand the impact of these sustainable agriculture management practices on the microbial diversity structure and composition. Such data are critical to evaluate the effect that these new strategies may have on microbial communities in the light of sustainable goals because microbial communities are crucial to maintain soil ecosystem functioning and to develop a sustainable agricultural model, based not only on crop productivity but also on ecological principles.

1.6.1 Soil types and plant genotypes

Many research studies have suggested that soil is the starting inoculum of microbiota from which plants select their microbiome. Therefore, soil type plays a vital role in microbiota selection as distinct soil types can have very different microbial community structures. However, the assessment of the extent to which soil type influences the microbial community is challenging to measure under field conditions as not only the soil characteristics but also the climate, cropping history or agricultural management can also influence the soil microbiome (Costa *et al.*, 2006, Costa *et al.*, 2007). Schreiter *et al.* (2014) studied the rhizosphere microbiome in the three distinct soil types which

were exposed for more than 10 years to the same climatic conditions and cropping history. In lettuce, they observed that the rhizosphere effect differed depending on the soil type that ultimately influenced the distinct bacterial composition. Bulgarelli *et al.* (2012) reported through pyrosequencing of 16S rRNA gene fragments amplified from total community DNA of *A. thaliana* that soil type explains the composition of root-inhabiting bacterial communities and plant genotype determine their ribotype profiles.

Plant genotypes are also a driving factor for microbiota selection. Different plant species and even different genotypes of one species, can possess different root architecture and exudation patterns, which strongly influence plant associated bacterial communities' structure and composition (Bais *et al.*, 2006, Bulgarelli *et al.*, 2013). Rybakova *et al.* (2017) studied the endophytic seed microbiome in *B. napus* and reported that three different cultivars of OSR shared only one third of the total 8362 OTUs, This shows the cultivar dependency and specificity of the OSR endophytic microbiome. Moreover, Lundberg *et al.* (2012) studied the microbiome of more than 600 *A. thaliana* plants and reported that rhizosphere and root endophytic microbiota are, to a large extent, dependent on host genotypes. They also observed different rhizosphere and endophytic bacterial communities in plants grown in two geochemically distinct bulk soils.

1.6.2 Soil tillage

Tillage is defined as the mechanical manipulation of the soil through which man can directly influence the soil for the purpose of crop production affecting significantly the soil physical, chemical and biological characteristics. It is well recognized that agricultural productivity strongly relies on a wide range of ecosystem services provided by the soil biota (Altieri, 1999). Although the delivery of ecosystem services are driven by complex interactions between the soil biota and abiotic parameters (Kibblewhite *et al.*, 2008), most soil processes related to organic matter transformation and nutrient cycling are mediated by microorganisms (Nannipieri and Badalucco, 2003). Moreover, microorganisms also contribute to soil aggregate formation and aeration, as well as carbon sequestration in agroecosystems (Six *et al.*, 2006).

Ploughing is one of the main components of conventional farming and has been used for centuries to control weeds, temporary alleviate soil compaction, and improve

nutrient mineralization and availability (Hobbs *et al.*, 2008). Besides these short-term benefits, long-term detrimental effects such as soil erosion, loss of soil organic matter, greenhouse gas emission, and disturbance in soil micro fauna have been recognized (Six *et al.*, 1999, Montgomery, 2007). Alternative soil management such as conservation reduced or no tillage practices can significantly enhance both soil quality and crop productivity in agroecosystems (Scopel *et al.*, 2013). In Irish cropping systems, conventional tillage associated with crop cultivation is currently the most commonly used tillage system employed by farmers. Conventional tillage refers to soil inversion down to approximately 25–30 cm using a mouldboard plough whereas under conservation reduced tillage the top 10 cm of soil is disturbed in order to improve the conditions for seed germination and >30 % of crop residues are left on the soil surface after planting (Vian *et al.*, 2009). Nowadays, there is a growing interest in using alternative tillage systems that minimize soil disturbance. Conservation tillage is less labour intensive and seems to be cost effective (Lahmar, 2010). Furthermore, tillage practices also influence the various abiotic factors such as pH, soil moisture, oxygen availability, quality of organic substrates, nutrient inputs such as nitrogen and phosphorus, soil texture and temperature, as well as biotic factors for instance the occurrence of other soil organisms such as earthworms (Six *et al.*, 1999, Young & Ritz, 2000, Busari *et al.*, 2015, Degrune *et al.*, 2017), which in turn may influence microbial communities and the ecosystem services they provide.

Dorr de Quadros *et al.* (2012) demonstrated through 16S amplicon metagenomic sequencing that soil microbial diversity, exchangeable P, Mg concentration, total organic carbon, nitrogen, mineral nitrogen and the anaerobic soil microbial community such as Verrucomicrobia, Firmicutes, Crenarchaeota, Chlamydiae, Euryarchaeota and Chlorobi were significantly higher in conservation tillage compared to conventionally tilled plots. Navarro-Noya *et al.* (2013) also observed similar results that under reduced tillage the microbial biomass and plant organic matter and nutrient content was higher. Jacobs *et al.* (2009) found that conservation tillage compared with conventional tillage not only improved aggregate stability but also increased the concentrations of soil organic carbon and nitrogen within the aggregates in the upper 5–8 cm soil depth after 30 years of tillage treatments. Yin *et al.* (2017) reported through amplicon metagenomic analysis that copiotrophic bacterial families such as *Oxalobacteriaceae, Pseudomonasaceae* and *Cytophagaceae* were more abundant in reduced tillage and

oligotrophic bacterial families such as *Gaiellaceae* and those within *Gemmatimonadetes* were more abundant in conventional tillage. However, Degrune *et al.* (2017) observed contradictory results. They found higher abundance of copiotrophic bacteria under conventional tillage and oligotrophic bacteria under reduced tillage. In either case tillage practices clearly alters soil microbial community structure, the response of individual groups appears to be very context-specific and cannot be generalized across various agroecosystems. The response is largely dependent on the soil physical and chemical conditions, soil types and climatic conditions. Despite the fact that under a specific edaphic and climatic context, different tillage regimes created different ecological niches that select for different microbial lifestyles with potential consequences for the ecosystem services provided to the plants and their environment.

1.6.3 Crop rotation

Another agriculture management practice that can impact on microbial diversity is crop rotation, the practice of growing different crops in succession on the same land. Diversified crop rotations benefits include increased soil nutrients and organic matter content (McDaniel *et al.*, 2014, Lehman *et al.*, 2017), enhanced soil-water use efficiency (Larkin *et al.*, 2010), stabilize the soil temperature, improved soil structure and tilth (Ball *et al.*, 2005), enhanced soil microbial biomass, diversity and activity (McDaniel *et al.*, 2014), and disruption of pests, weeds and disease cycles (Peters *et al.*, 2003, Hopkins *et al.*, 2004). Some of these rotational effects result from variation in root architecture and rhizodeposition, biogeochemical cycles, host plant identity and genetics, soil characteristics and climatic conditions, that collectively also influence the size, structure and dynamics of soil and plant-associated microbial communities (Garbeva *et al.*, 2004, White *et al.*, 2013, Lehman *et al.*, 2015). Hooper *et al.* (2000) stated that increasing aboveground crop biodiversity can result in corresponding increases in diversity belowground. Additionally, crop rotation may be the best, most widely practiced, and most cost-effective method for reducing soil borne fungal pathogen populations.

For instance, *B. napus* rotated with tobacco supress the tobacco black shank (*Phytophthora parasitica* var. *nicotianae)* fungal disease by secreting antimicrobial compounds such as 2-butenoic acid, benzothiazole, 2-(methylthio) benzothiazole, 1-(4-

ethylphenyl)-ethanone, and 4-methoxyindole from the roots (Fang et al., 2016). Plant roots can continuously produce and secrete many compounds into the rhizosphere to mediate the interactions between the roots and pathogens (Bais *et al.*, 2004). Pathogens can recognize the signals in the root exudates to colonize the host plant (Bais *et al.*, 2004). Plant roots can also secrete a number of substances to protect themselves against pathogen and non-host pathogen infection (Bais *et al.*, 2006). Moreover, OSR is also an effective break crop for cereals to inhibit the take-all fungus infection in wheat crop (Angus et al., 1991). Zhao et al. (2017) compared the endosphere microbiome of symptomatic and asymptomatic *B. napus* roots infected with *Plasmodiophora brassicae* (a causal agent of clubroot disease) through 16S amplicon high throughput sequencing. They observed that the bacterial population and its relative abundance were far higher in the asymptomatic roots than symptomatic OSR root samples. For instance, the bacterial families *Oxalobacteraceae, Pseusomonadaceae, Comamonadaceae, Rhizobiaceae, Xanthomonadaceae, Flavobacteriaceae, Sphingobacteriaceae,* and *Methylophilaceae* were significantly higher in asymptomatic roots of OSR under *P. brassicae* infection and, many of these microorganisms are known for their biocontrol and PGP functions. It was shown several decades ago that disease suppressive properties of soil were largely induced by long-term cultivation of monoculture leading to build up of host specific microbial communities (Whipps, 1997, Kwak & Weller, 2013, Hegewald et al., 2017). The specific roles of the PGP bacteria in development of the disease suppressive traits was rarely addressed in the studies on disease suppressive soil communities; however, bacteria of genus *Streptomyces, Bacillus, Actinomyces, Pseudomonas* that lead a rhizospheric endophytic lifestyle were shown to contribute to the disease suppressive traits of soils (Raaijmakers & Weller, 1998, Weller et al., 2002, Mendes et al., 2011).

1.6.4 Effects of fertilizer and pesticide application on OSR microbiota

Modern agriculture largely depends on the widespread application of agrochemicals, including inorganic fertilizers and pesticides. Indiscriminate, long-term and over-application of these chemicals have severe effects on soil ecology that may lead to alterations in or the erosion of beneficial or plant and soil probiotic microflora (Kalia & Gosal, 2011). Field-grown plants could be exposed to these detrimental chemicals either intentionally (e.g. by spraying) or from residues remaining from previous

applications (Khan *et al.*, 2004). These chemicals or their degraded products interact with soils and plant associated microbes, and cause DNA, protein, oxidative or membrane damage (Pham *et al.*, 2004), they can adversely affect N_2 fixation (Anderson *et al.*, 2004), protein synthesis and metabolic enzyme activity (Boldt & Jacobsen, 1998), they can disrupt the signalling between the microbes and plant phytochemicals (Fox *et al.*, 2007), reduce the photosynthate allocation (Datta *et al.*, 2009), or can restrict root growth (Eberbach & Douglas, 1989). Additionally, pesticides that persist in soils may have a long lasting impact on rhizobia survival and function.

Fox *et al.* (2007) found that pesticides disrupt the signalling between legume plant derived phytochemicals (luteolin, apigenin) and *Rhizobium* Nod D receptors which are essential for nodulation and symbiotic nitrogen fixation. However, plant growth promoting bacteria (PGPB) including symbiotic N_2 fixers can affect plant development either indirectly by circumventing the toxic effects of pesticides or directly by synthesizing the plant growth regulating substances (Jeon *et al.*, 2003). Inoculation of fipronil and pyriproxyfen insecticide tolerant and phytohormone producing *R. leguminosarum* strain MRP1 promoted the growth of pea when it was applied as seed inoculant in fipronil and pyriproxyfen treated soil (Ahemad & Khan, 2010). Germaine *et al.* (2006) inoculated pea plant with a *Pseudomonas* endophyte capable of degrading the organo-chlorine herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D). When inoculated plants were exposed to 2,4-D, they showed no accumulation of the herbicide into their tissues and experienced little or no signs of phytotoxicity, whereas un-inoculated plants showed significant accumulation of 2,4-D and displayed signs of toxicity including a reduction in biomass, leaf abscission and callus development on their roots. Diazotrophic microorganisms perform biological nitrogen fixation (BNF) and possess the genes involved in the synthesis and functioning of the nitrogenase (*nif*, N_2 fixation) enzyme (Souza *et al.*, 2015). It was demonstrated that the colonization ability of the nitrogen-fixing endophytic bacterium *Acetobacter diazotrophicus* was significantly decreased in the sugarcane plants fertilized with high levels of nitrogen (Fernandez *et al.*, 2012). Analysis of the endophytic population of maize roots under treatment with herbicides and different fertilizer types revealed that differentiated microbial communities developed in maize plants cultivated using mineral fertilizer, compared to plants cultivated using organic fertilizer (Seghers *et al.*, 2004). These

studies did not reveal if the changes in endophyte population were a consequence of changes in overall soil microbial population upon the fertilizer and pesticides treatment.

The plant microbiomes have been extensively studied so far due to their exceptional valuable impact on plants; however the tripartite interaction between agriculture management practices, the microbiota and the host plant is yet poorly understood. Several plant growth promoting microbes are commercialised for their application as microbial inoculants such as bio-fertilizer, plant straighteners, biocontrol agents, bio-pesticides, and phytostimulants (Berg, 2009). However, the efficacy of many microbial inoculants is often not observed at field scale due to issues with the establishment of the biological agent, as they have to face competition with the native soil microbiota (Le Cocq *et al.*, 2017). Moreover several aforementioned influencing factors as well as environmental factors shape the plant microbiome. Therefore, further in-field research is necessary to investigate the impact of agricultural management practices as a stimulants for the indigenous bacterial communities to respond and support host plant growth and defence. The advancement of meta-omic approaches has made it assessable to explore the plant-microbiome in-detail, and will further allow researchers to inspect the practices that manipulate the plant microbiota and their intrinsic traits in order to test interactions.

Chapter 2

Research Objectives

2.1 **Aims of this research**

Recent soil/plant microbiome research advancements, utilising rapid-high-throughput sequencing technologies, strengthen a better understanding of complex plant–associated microbial communities, their interactions, and harness them to increase productivity, improve plant health and mitigate effects of climate change (Ahkami et al, 2017; Finkel et al., 2017 Berendsen et al., 2012). Altering these soil/plant microbiomes could promote the environmentally benign agriculture, for instance, enriching soil with microbes such as *Pseudomonas*; known for their plant growth promotion and effective disease suppression (Mauchline & Malone, 2017; Lally et al, 2017, Oteino et al, 2015), they would promote sustainable agriculture. Agricultural management practices play a crucial role in transforming the soil and the subsequent phytobiome (Finkel *et al.*, 2017). Microbiome studies of economically important OSR crop are continually being reported, however the implications of different management practices on the microbiome of OSR, remain to be explored.

Traditionally, OSR is cultivated under conventional plough tillage (CT) in Ireland, but changing OSR crop establishment and management practices from conventional to conservation strip tillage (ST) raised three major questions: (i) what will be the impact on soil and plant associated microbial communities?, (ii) can it reduce the greenhouse gases emission?, and (iii) will it still provide adequate crop production? A large comprehensive research project was designed leading whereby this hypothesis was mainly focused on studying the impact of CT vs ST practices on winter OSR-associated microbiome under Irish agronomic conditions. To this end, the following four research objectives are proposed:

Objective 1: Investigate the effect of tillage (CT vs ST) practices on winter OSR (WOSR) microbiota structure and composition at plant maturity (Chapter 3).

The plant microbiome is compartmentalized into its rhizosphere, endosphere, and phyllosphere microbiota, where soil largely being the original source of microbial diversity. This is observed through different plant microbiome research studies such as *Arabidopsis*, maize, rice, and barley microbiome (Lundberg *et al.*, 2012, Peiffer *et al.*, 2013, Bulgarelli *et al.*, 2015, Edwards *et al.*, 2015). Previous studies reported that tillage practices alter the physiochemical soil properties and bring about changes in

soil microbiota (Vian *et al.*, 2009). However, experimental validation of soil tillage impact on endophytic microbiota is under studied. The goal of this research was to obtain an in-depth understanding of the structure and composition of the bacterial microbiota associated with WOSR. The composition of WOSR microbiota, if, influenced by CT and ST practices at plant maturity stage in four compartments was characterized. Building on this, it was hypothesised that the two tillage practices could influence microbial allocation differently in WOSR.

Objective 2: Evaluate the effects of soil tillage practices (CT vs ST) on rhizosphere microbiome at different plant growth stages of WOSR (Chapter 4).

The rhizosphere is considered as the hotspot of plant-microbe interactions and the dynamics of the rhizobiome alters with changes in rhizodeposition over the plant life cycle, and changes in soil characters may also alter the rhizobiota (Mendes *et al.*, 2013). Thus, the aim of this work was to explore the niche separation of rhizosphere from the bulk soil under the influence of plant developmental stages and determine the tillage-dependent effects on the WOSR rhizosphere microbiota. It was hypothesised that WOSR rhizosphere microbial communities would change from the bulk soil as the crop develops and soil tillage fine-tunes the bacterial composition.

Objective 3: Explore the structural variability and niche differentiation in the bacterial microbiome of WOSR rhizosphere and endosphere at different plant growth stages under the influence of soil tillage (Chapter 5).

Niche differentiation of the plant microbiome at the rhizosphere soil-root interface and their alteration with plant development has been reported in a limited number of studies. However, each microenvironment (rhizosphere, root, shoot) provides specific biotic and abiotic conditions for microbial life. Indeed, Bulgarelli *et al.* (2013) suggested that an additional fine-tuning and niche differentiation of microbiota exist in the aerial plant organs, and Beckers *et al.* (2017) recently reported the bacterial niche differentiation in shoot compartment along with rhizosphere and root microhabitat in Poplar trees. Further investigations to validate this concept with agronomic parameters are required for different crops. Hence, the goal of this research work was to study the influence of soil tillage (CT vs ST) on structure and

composition of the WOSR bacterial microbiome and niche differentiation at different plant developmental stages. The hypothesis of this chapter claimed that structure, composition and diversity of bacterial communities in WOSR associated compartments (rhizosphere, root and shoot) would change with plant development, and soil tillage would be the driving factor for WOSR microbiome variation.

Objective 4: Examine the impact of OSR-wheat crop rotation in combination with soil tillage (CT vs ST) on the prevalence of 2,4-diacetylphloroglucinol (2,4-DAPG) producing fluorescent *Pseudomonas* spp. inhabiting the rhizosphere and root microhabitats using quantitative real time PCR (Chapter 6).

Previous reports highlighted the importance of 2,4-DAPG producing fluorescent pseudomonas spp. in disease suppression (Raaijmakers & Weller, 1998). Despite their potential role in eco-friendly crop production, the impact of agriculture management practices (CT, ST and crop rotation) on 2,4-DAPG producing bacteria in soil (Picard & Bosco, 2006, Rotenberg *et al.*, 2007, Meyer *et al.*, 2010, Raaijmakers & Mazzola, 2012) however requires further exploitation specifically in the rhizosphere and root microhabitats. Therefore, the aim of this experimental chapter was to evaluate the prevalence of 2,4-DAPG producing *Pseudomonas* spp. in the rhizosphere and roots of WOSR and wheat crops, at different plant growth stages under a crop rotation regime and two CT *vs* ST tillage practices, using qRT-PCR technique, over two successive years (2015 and 2016). The working hypothesis was that crop rotation of a wheat crop with WOSR would lead to significant differences in prevalence of 2,4-DAPG^{+} *Pseudomonas* spp. between CT and ST tillage practices throughout the plants life cycle.

Chapter 3

Crop establishment practices are a driver of the plant microbiota in Winter Oilseed Rape (*Brassica napus* L.)

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3.1 **Introduction**

Soil is the foundation of productive agriculture and represents the most diverse and significant ecosystem on Earth (Roger-Estrade *et al.*, 2010). The collective microbial community in soil, referred to as the microbiota, underpins many soil ecosystem functions (Kibblewhite *et al.*, 2008) regulating soil fertility, biogeochemical cycling and impacting on plant performance (Fierer *et al.*, 2012). For instance, the host plant is assisted by its microbiota in nutrient acquisition, phytohormone production, phytotoxic compound degradation, tolerance to biotic and abiotic stress and the suppression of pathogens (Whipps, 2001, Compant *et al.*, 2005, Glick, 2012). In return, the plant provides a favourable environment for microbial growth and a continuous supply of carbon rich rhizodeposition (Zhang *et al.*, 2009). Consequently, and similar to other eukaryotic organisms, plants can be considered holobionts whose growth, development and health are ultimately determined by the outcome of host-microbiota interactions (Bordenstein & Theis, 2015). In this respect, many research studies have shown that plant-microbe interactions are not only essential for developing an understanding of plant growth and health, but are of considerable importance with respect to developing sustainable agricultural practices (Berg *et al.*, 2014).

Agricultural management practices influence soil physical, chemical and biological properties, which have direct impacts on soil microbial composition and behaviour (Jangid *et al.*, 2008). Conventional tillage practices invert the soil to a depth of about 20-35 cm through ploughing, and leave <30 % of crop residues on the soil surface. The mechanical disruption of soil leads to water and nutrient losses, soil erosion, soil degradation due to low organic matter content and a fragile soil structure (Vian *et al.*, 2009). Shifting conventional tillage to conservation tillage practices such as strip tillage, significantly reduces these impacts (Hobbs *et al.*, 2008). Conservation tillage generally encompasses shallow working depths without soil inversion and retains >30 % of crop residues on soil surface which, over a number of years, helps to maintain soil moisture, increases soil organic matter content, reduces soil erosion, promotes soil fertility and biological activity (Vian *et al.*, 2009). However, in oceanic/temperate regions, conservation tillage presents challenges from a weed control perspective and crop establishment can be difficult in wetter conditions with slower early crop growth.

Tillage practices have been shown to influence microbial community structure, taxonomic composition, microbial abundance and activity by changing the physicochemical properties of soil (García-Orenes *et al.*, 2013). For instance, Zhang *et al.* (2012) reported that microbial biomass accumulation was tillage dependent and recommended conservation tillage as an effective component to improve soil quality and sustainability. Smith et al. (2016) showed that there was a significant difference in the soil microbial community structure and predicted function as a consequence of conventional tillage or no-tillage systems. For instance, bacterial populations carrying genes involved in protein degradation, ammonia assimilation and denitrification were higher in the no-tillage system, while bacterial populations carrying genes involved in ammonification and nitrous oxide production were higher in conventional tilled soils. Zhang *et al.* (2012) showed that phospholipid fatty acid (PLFA) profiles and soil enzyme activities were significantly higher in no tilled soils than in ridge tilled soils.

OSR is the world's third largest source of vegetable oil (USDA-FAS, 2015) used in human nutrition and as a source of oil for biodiesel production. OSR is grown as spring and winter OSR varieties. WOSR is also an important break crop in cereal crop rotation and can significantly reduce the rate of 'take-all' fungal disease (*G. graminis var. tritici*) as a result, improves the yield of subsequent cereal crops (Angus *et al.*, 1991, Hilton *et al.*, 2013). Although several studies provided insights into host-microbiota interactions in OSR (Germida *et al.*, 1998, Macrae *et al.*, 2000, Kaiser *et al.*, 2001, Hilton *et al.*, 2013), they generally utilised low-resolution, analytical protocols which makes it difficult to develop a fundamental understanding of the significance of these microbes to OSR production. For instance, the impact of soil tillage on the microbiota of OSR, and the potential implications for crop production, remains largely unknown.

The aim of this research was to obtain detailed knowledge of (a) the composition of the bacterial microbiota associated with WOSR and (b) how this composition is influenced by conventional plough and conservational strip tillage practices. In particular, we were motivated to test the hypothesis that different WOSR microhabitats (rhizosphere, root and shoot) host distinct microbiota whose composition is modulated by tillage practices.

3.2 Materials and Methods

3.2.1 Experimental design

The plant and soil samples for this study were taken from a field experiment evaluating the effect of crop establishment systems on the growth and development of WOSR. The establishment systems comprised of, (1) a conventional plough based system (CT) and (2) a low-disturbance conservation strip tillage system (ST). The conventional establishment system comprised of mouldboard ploughing which inverted the soil to a depth of 230 mm, two days prior to sowing. The ploughed soil then received secondary ploughing to 100 mm depth with a rotary power harrow and the WOSR was sown at 10 mm depth at row spacing of 125 mm using a conventional mechanical delivery seed drill operated in combination with the power harrow. The strip tillage establishment system deployed was a non-inversion system, comprised of a single cultivation/seeding pass of a rigid leg cultivator with legs spaced at 600 mm apart which were operated at 200 mm depth. These forward facing tines, with side 'wings' giving additional soil disturbance, worked directly in the cereal residue of the previous crop, disturbing approximately 50 % of the surface width between the legs. This was the first year that strip tillage was used in this field, as in previous years plough based tillage practices had been used. Seeding was by metered pneumatic delivery of seed to a point behind the cultivator leg, giving a row spacing of 600 mm. For the microbiota studies, plant and soil samples were taken from these two establishment systems (CT and ST) in three replicated plots. The trial was a randomised block design with individual plot dimensions of 24 m x 4.8 m and was located in an area known locally as the sawmill field at the Teagasc Crops Research Centre, Oak Park, Carlow, Ireland (52.857478 ^{o}N, -6.922467 ^{o}W). The previous crop was winter barley and cereal crops had been sown for more than 5 years previously. The WOSR variety 'Compass' was sown at a seed-rate of 60 seeds/m^2 on 28th August 2013 in both establishment systems. Subsequent to seeding, the soil surface was rolled using a ring roller. The top soil was a well-drained sandy loam overlying inter-bedded layers of sand, gravel and silt/clay. The top soil had a sand content of 50-70 % with less than 20 % clay. Physical and chemical characterisation of the soil substrates used in this study described in Table A3.1. Crop management, other than crop establishment, followed standard practices for

WOSR production in this region. A pre-emergence selective herbicide (quinmerac and metazachlor) was applied post seeding for weed control. The crop received two fungicide applications (prothioconazole) in October and March for phoma stem canker (*Leptosphaeria sp.)* and light leaf spot (*Pyrenopeziza brassicae*) control. Phosphate (P) and potassium (K) fertiliser was applied on the basis of soil test results post sowing according to Teagasc guidelines (Coulter *et al.*, 2008). Fertiliser N (a total of 225 kg N ha^{-1}) was applied in three equal applications at 2 week intervals starting in late February.

3.2.2 Sample collection of bulk soil, rhizosphere and plant fractions

Bulk soil and plant samples were collected from the two treatments; conventional tillage (CT) and conservation strip tillage (ST) in triplicate from three replicate plots per treatment at the harvesting stage (~330 days after sowing). Bulk soil samples were collected from a depth of 0-25 cm, in triplicate from the edges of each plot, using a hand auger. For each plot, composite soil samples were prepared by thoroughly mixing the triplicate samples and a representative subsample of this was collected in sterile 50 mL Falcon tubes. The plant samples were processed into three plant microhabitat zones i.e. rhizosphere soil, root and shoot. The excess soil from the root was removed by manual shaking, leaving ~1 mm of rhizosphere soil still attached to the root. The rhizosphere soil attached to the root was scraped off with a sterile forceps into sterile 50 mL Falcon tubes. The root and shoot samples were washed separately in 50 mL Falcon tubes containing 30 mL of Phosphate Buffered Saline (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 7.0 pH, 0.02 % Silwet L-77) to remove the tightly adhered microbes followed by a sonication step (30 s at 50-60 Hz) as described by Lundberg *et al.* (2012). Root and shoot samples were frozen using liquid nitrogen and stored in pre-labelled sterile 50 mL Falcon tubes. All the samples were stored in -80 ^oC until required for DNA extraction.

3.2.3 DNA extraction from soil and plant microhabitat zones

DNA extractions were performed on 3 bulk soil, 3 rhizosphere soil, 3 root and 3 shoot samples per plot with 3 plots per treatment (CT and ST). For DNA extraction in soil, 0.25 g of soil was taken individually from each composite soil sample and

processed according to the protocol from MoBio PowerSoil^{TM} DNA isolation kit (Carlsbad, CA, USA). Total soil DNA was eluted in 50 µL of sterile water (Sigma Aldrich). For DNA extraction from the plant samples, 0.5 g of plant tissues were individually ground in liquid nitrogen. The DNA was extracted following 2 % cetyl trimethylammonium bromide (CTAB) method described by Doyle (1990). Total plant DNA was eluted in 100 µL of sterile water. Concentration and purity of DNA was determined by Nanodrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA). Post quantification, all DNA samples were normalised to 10 ng μL^{-1}. The three DNA samples from each microhabitat zone per block were pooled (e.g. the three DNA samples from the shoot samples from block 1 were pooled) to give representative DNA samples of bulk soil, rhizosphere, root and shoot from each block.

3.2.4 Illumina sequencing of 16S rRNA gene amplicon libraries

The amplicon library of bacterial DNA was generated using the PCR primers: 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'), 785R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'), with Illumina adapter overhang sequences (underlined) which covered ~464 bp of the hypervariable regions V3 and V4 of the 16S rRNA gene (Klindworth *et al.*, 2013). Amplicons were generated, purified, indexed and sequenced with some modifications according to the Illumina MiSeq 16S Metagenomics Sequence Library Preparation protocol (16S-Metagenomic-library-prap, 2013). An initial PCR reaction contained 25 µL of 2 x KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), 1 µL of forward primer (1 µM), 1 µL of reverse primer (1 µM), 2.5 µL of DNA (~10 ng μL^{-1}) and 20.5 µL of nuclease free H_2O in a total volume of 50 µL. The PCR reaction was performed on a 96-well Thermocycler using the following program: 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec and a final extension step at 72 °C for 5 min. All amplicons were cleaned using Ampure DNA capture beads (Agencourt- Beckman Coulter; Inc.) following addition of Illumina sequencing adapters and dual-index barcodes to each amplicon with the Nextera-XT Index kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The amplicon libraries were pooled in equimolar

concentrations. The final library was paired-end sequenced at 2 x 300 bp using a MiSeq Reagent Kit v3 on the Illumina MiSeq platform. Sequencing was performed on the Next Generation Sequencing Platform at Teagasc Moorepark research centre, Fermoy, Cork, Ireland.

3.2.5 Amplicon data analysis

16S rRNA gene sequences were analysed using Usearch v8.1 64 bit (www.drive5.com) (Edgar, 2013) and Qiime, v1.9.0 (Quantitative Insight into Microbial Ecology) (Caporaso *et al.*, 2010), unless otherwise specified the default parameters were used. Paired-end reads were merged using the command fastq_mergepairs in USEARCH by specifying a minimum overlap of 16 bp. Barcode sequences were removed from the merged paired-end sequences using the command extract_barcodes.py in QIIME. We used USEARCH to demultiplex the pre-processed sequencing reads and to generate a quality report. We used the fastq_filter function in USERACH to truncate all the reads to a length of 400 bp and discard sequences shorter than this length and sequences that contained more than 4 expected base errors per read. The retained high-quality sequencing reads then clustered into operational taxonomic units (OTUs) at 97 % sequence identity using the USERACH pipeline. Singletons were discarded from further analysis and the "Gold" reference database (http://drive5.com/uchime/gold.fa) was used to identify and remove chimeras. Taxonomic classification of OTU-representative sequences was performed in QIIME using RDP (Ribosomal Database Project) classifier (Wang *et al.*, 2007) trained against the Greengenes database (DeSantis *et al.* (2006), release 13_5). Likewise, we used OTU representative sequences to generate a phylogenetic tree in QIIME using 'muscle' as alignment method. The generated OTU table, taxonomy information and phylogenetic tree were used to implement the ecological and statistical analyses.

3.2.6 Statistical analysis

Due to the intrinsic complexity of our experimental design, contemplating field sampling, we decided to use a dedicated kit for the preparation of soil-derived (i.e., soil and rhizosphere) and plant-derived (i.e., root and shoot) specimens. Therefore, the differences in DNA preparation could contribute, at least in part, to apparent differences in the WOSR microbiota composition. For this reason, we generated two independent datasets for the data analysis: one comprised of soil-derived microhabitats (bulk soil and rhizosphere samples) and another containing plant-associated microhabitats (root and shoot). Data analysis and visualization were performed using Phyloseq (McMurdie & Holmes, 2013) package from R operated through R Studio v0.99.893. All OTUs belonging to chloroplast and mitochondria were identified and removed from the data set prior the analysis. To assess differential bacterial abundance between the samples, we used ANCOM (Analysis of Composition of Microbiomes) (Mandal *et al.*, 2015), a statistical test developed for microbial count data, using R with additional parameters multcorr = 2 and sig = 0.05, that is with multiple testing correction at significance 0.05. For alpha diversity analysis, observed OTUs, Chao1 and Shannon indexes, normal distribution of the data were checked with the Shapiro-Wilk test. Significant differences in the variance of parameters were evaluated, depending on the distribution of the estimated parameters, either with parametric t-test or non-parametric Mann-Whitney-Wilcoxon and Kruskal-Wallis tests to identify significant differences between the two tillage systems and microhabitat zones. Post hoc comparisons were conducted by Kruskal-Wallis Dunn test. For such analysis, sequencing reads of soil samples (bulk soil and rhizosphere) and plant samples (root and shoot) were rarefied at an even sequencing depth 6,191 and 9,765 reads/sample respectively. To compare community diversity between the samples (beta-diversity), Principal Coordinate Analysis (PCoA) based on Bray-Curtis, sensitive to OTU abundances, and Weighted UniFrac, sensitive to OTU abundances and taxonomic affiliation, distances were calculated by using counts per million transformed OTU abundances. Permutational multivariate analysis of variance using distance matrices was performed in R using the 'adonis' function to define the proportion of variance explained by the factors microhabitat and/or tillage. A differential analysis of the OTUs relative abundances using moderated shrinkage estimation for dispersions and fold changes as an input for a pair-wise Wald test was

carried out in DESeq2 package from R v1.14.1 (Love *et al.*, 2014). This test identifies the number of OTUs significantly enriched in different compartments corrected for tillage practices, and in two tillage practices corrected for individual compartment with an adjusted *P* value (False Discovery Rate, FDR $P < 0.05$). We used a Venn diagram to visualise enriched OTUs, unique and shared, in root and shoot microhabitat zones under CT and ST.

3.3 **Results**

3.3.1 General characterisation of the WOSR microbial communities

The microbiota of WOSR grown under two cultivation systems, CT and ST, were analysed at maturity (harvesting stage). 16S rRNA sequencing libraries of the bulk soil, rhizosphere soil, roots and shoots were prepared and sequenced. The analysis generated 992,256 sequence reads of which 691,230 (~69.64 % total sequence reads) were retained upon quality-filtering. However, these PCR primers were incapable of discriminating between plant-derived (e.g. plastids) and microbial-derived 16S rRNA gene sequences. Therefore, we reasoned that the first step in the data analysis was to identify potential host plant-derived 'contaminants' in our dataset (Table A3.2). Indeed, while the proportion of plant-derived sequences in bulk soil and rhizosphere samples were negligible (<1 %), approximately half of the root and shoot-associated reads were identified as plant derived sequences (Table A3.2, Figure A3.1). Upon *in silico* removal of these sequences, we were able to retain enough high quality reads per sample (max=65,113, min=6,191, median=28,801). These sequencing reads were clustered using >97 % sequence similarity to prokaryotic operational taxonomic units. The total numbers of microbial OTUs was 2,161 (Table 3.1). Rarefaction curves based Chao1 analysis showed OTU saturation at ~15,000 sequence reads per sample (Figure A3.2).

Table 3.1 Quality metrics for sequencing data.

Total number of reads and read lengths				
Total number of raw reads before QC	992,256			
Total number of assigned reads after QC	691,230			
Average read length after QC	400 bp			
Assigned reads	**Bulk Soil**	**Rhizosphere soil**	**Root**	**Shoot**
Average number of reads	29070 ± 12264	32780 ± 20153	42902 ± 16005	60669 ± 1995
Non-target reads (%)	0.25 ± 0.04	0.27 ± 0.10	49.2 ± 5.99	46.56 ± 5.84
Average number of assigned reads	28996 ± 12231	32700 ± 20115	21891 ± 7967	31618 ± 7834
Normalised reads per sample	6191	6191	9765	9765
Average number of assigned OTUs	969 ± 48	962 ± 114	438 ± 115	150 ± 16
Unclassified reads (%)	0.21	0.16	0.09	0.03
% of Total useable reads	25.2	28.4	19.0	27.4

3.3.2 Taxonomic assemblages of bacterial microbiota

Approximately 99 % of WOSR microbiota was represented in the top ten most abundant bacterial phyla (Figure 3.1). In particular, the phyla Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria, Verrucomicrobia and Chloroflexi largely dominate the bulk soil and rhizosphere soil microbiota. At the phylum level, bacterial communities of bulk soil and rhizosphere were very similar under CT. However, sequences assigned to phylum Bacteroidetes discriminated bulk soil (5.92 %) from the rhizosphere (14.92 %) profiles under ST. The phylum Bacteroidetes was more abundant in shoot communities in both tillage systems which distinguished the shoot from the root microbiota. There was a marked enrichment of the phylum Firmicutes (12.93 %) and depletion of phylum Actinobacteria (7.98 %) in root microbiota of ST compared to root under CT (Firmicutes 1.91 %; Actinobacteria 22.40 %). The shoot under both tillage practices contained very few microbes assigned to phylum Firmicutes. These results highlight a shift in community composition which progressively differentiated the root and shoot bacterial assemblages, from the soil biota; and whose magnitude is influenced, at least in part, by the tillage regime. Moreover, the ANCOM analysis showed that the abundance of 10 bacterial communities at phylum level; Acidobacteria, Armatimonadetes, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, Nitrospirae, Planctomycetes, Proteobacteria, and WS3 were significantly ($P < 0.05$) different in the bulk soil and each compartment under both tillage regimes; CT and ST (Figure A3.3).

Our results showed that in WOSR, tillage practice had a marked effect on rhizosphere, root and shoot microbiota but surprisingly, not on bulk soil microbiota. The WOSR bacterial composition at family level showed that, in the bulk soil 77 % of the OTUs had less than 1 % relative abundance. Families such as *Chthonionbacteraceae* (4 %), *Hyphomicrobiaceae* (2.9 %), *Bradyrhizobiaceae* (2.8 %) and *Bacillaceae* (2.8 %) were among the most abundant groups present in the bulk soil (Figure A3.4 & A3.5A). In the rhizosphere samples, 72 % of the microbiota were present in abundances of less than 1 % of the total population. Here families such as *Sphingomonadaceae* (5 %), *Sphingobacteriaceae* (3.5 %), *Micrococcaceae* (3.6 %) and *Chthoniobacteracaea* (3 %) were among the most abundant groups (Figure A3.5B). In the roots of WOSR 39 % of the microbiota

existed as less than 1% of the total root population. In the roots, *Pseudomonadaceae* were the most abundant family observed, making up 14 % of the total OTU count. This was followed by families such as *Sphingobacteriaceae* (9 %), *Bacillaceae* (2.8 %), *Xanthomonadaceae* (5 %) and *Flavobacteriaceae* (4 %) (Figure A3.5C). Finally, in the shoots, 23 % of the OTUs were present as less than 1 % of the population. This microhabitat appears to have a very different set of dominant microbes originating from families such as *Sphingobacteriaceae* (12 %), *Nocaridiaceae* (9.6 %), *Flavobacteriaceae* (8.6 %) *Rhizobiaceae* (8 %) and *Enterobacteriaceae* (6 %) (Figure A3.5D).

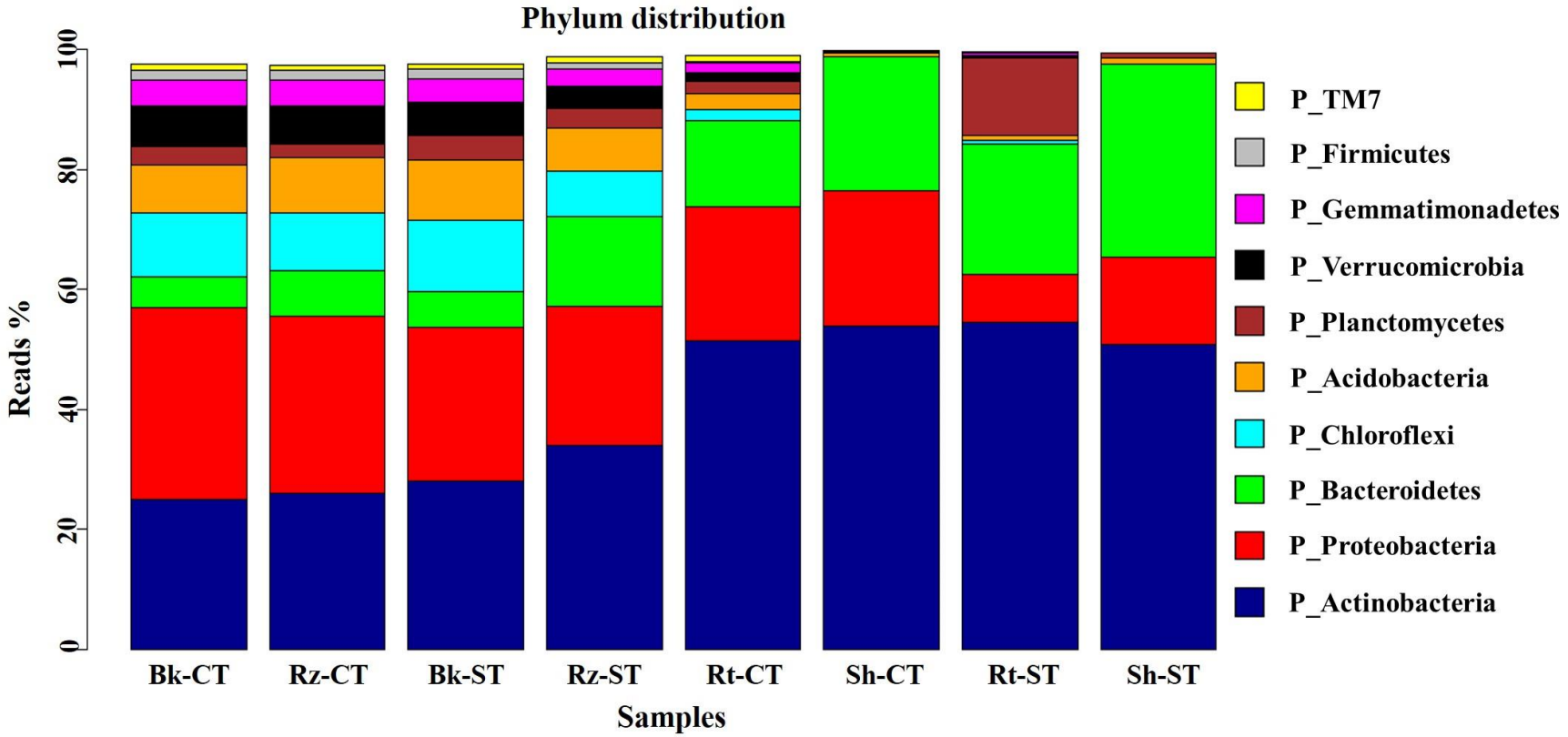


Figure 3.1 Phylum distributions of the OTUs. Average relative abundance (% of sequencing reads) of ten most abundant prokaryotic phyla associated with bulk soil (Bk), rhizosphere (Rz), root (Rt) and shoot (Sh) microhabitat zones of WOSR under conventional tillage (CT) and conservation strip tillage (ST), are displayed in different colours. For each sample type, the number of replicates are n = 3.

3.3.3 Bacterial alpha-diversity and beta-diversity

We investigated the effect of the tillage and compartment on microbiota composition at the OTU level, which represent the highest taxonomic resolution achievable in our investigation. Alpha diversity, the microbial diversity within each sample, was analysed based on the OTU richness, Chao1 and Shannon diversity indices (Figure 3.2). To control for differences in sampling effort across microhabitats, we rarefied the soil samples (bulk soil and rhizosphere) to 6,191 and plant samples (root and shoot) to 9,765 reads per sample before calculating the diversity indices. OTU richness was highly dependent on microhabitat type, with high richness values for bulk soil (969 ± 48) and rhizosphere soil (962 ± 114), and consistently decreased in richness estimates in the root samples (438 ± 115) and shoot samples (150 ± 16) (Table A3.4). For diversity and evenness estimates, the soil samples failed to identify tillage as well as compartmental effects on the WOSR microbiota (t-test; Mann-Whitney-Wilcoxon test; Kruskal-Wallis test; $P > 0.05$, Figure 3.2A, B & C, Excel sheet_WS-1). On the other hand, the plant microhabitatsm, root and shoot also failed to show a tillage effect (Mann-Whitney-Wilcoxon test; $P > 0.05$, Figure 3.2D, E & F, Excel sheet_WS-1). However, there was clear compartment effect observed in the WOSR plant samples (Kruskal-Wallis and Dunn's post-hoc tests, $P < 0.05$, Benjamini-Hochberg corrected). The soil samples displayed a greater richness and diversity compared to that of plant samples (Figure 3.2). In particular, the Shannon index showed a marked difference between the root samples of both tillage (CT and ST) practices (Figure 3.2F). Thus, the WOSR microbiota emerged as a progressively gated community whose composition appears largely defined by the plant microhabitat type.

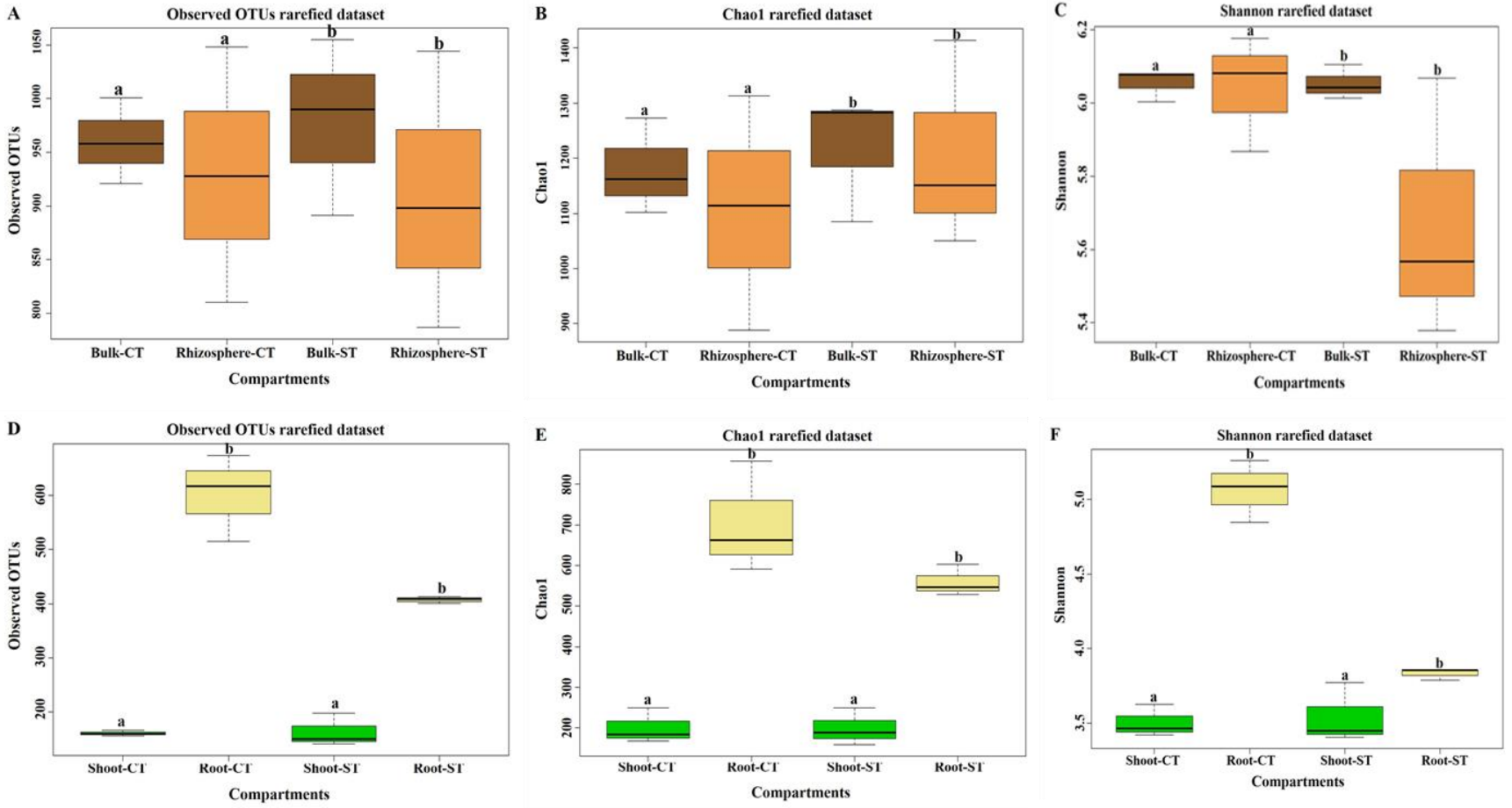


Figure 3.2 Variation patterns of alpha diversities of the bacterial communities associated with bulk soil, rhizosphere, root and shoot under two tillage practices; CT and ST. The alpha diversity estimates; Total number of observed OTUs, Chao1 estimator and Shannon's diversity of soil samples (bulk soil and rhizosphere) are displayed in A, B and C respectively, and of plant samples (root and shoot) are displayed in D, E and F respectively. Sequencing reads of soil samples and plant samples were rarefied at an even sequencing depth

6,191 and 9,765 reads/sample respectively prior the analysis. *Lowercase letters* denote statistically significant differences by Kruskal–Wallis and Dunn's post-hoc tests, $P < 0.05$, Benjamini-Hochberg (BH) corrected between the plant compartments within one tillage system. Statistical results of alpha diversity are displayed in Excel sheet_WS-1.

To elucidate whether the composition of bacterial communities correlated with, the microhabitat and/or tillage system, we used the OTU count data to construct dissimilarity matrices with Bray–Curtis (BC), sensitive to OTUs relative abundance (Bray & Curtis, 1957) and Weighted UniFrac (WUF), sensitive to OTUs relative abundance and taxonomic relatedness (Lozupone *et al.*, 2011). These matrices were visualised using Principal Coordinate Analysis (PCoA) as shown in Figure 3.3. At the OTU level, PCoA analyses revealed a clear separation between the root and shoot microhabitats and to a lesser extent between the bulk soil and rhizosphere microbiota. Partitioning of variance (ADONIS) based on BC distance matrix (Figure 3.3A & B, Excel sheet_WS-2) of soil samples (bulk soil and rhizosphere) indicated minor contribution of the soil microhabitat type ($P = 0.05$) and showed no influence of tillage practices ($P > 0.05$). However, WUF analysis of the soil samples showed a significant contribution of microhabitat type and tillage methods to the clustering of WOSR soil microbiota. ADONIS based on BC distance matrix and WUF analysis showed that plant microhabitat type (root or shoot), tillage practice, and their interactions had significant contributions to the differentiation of the root and shoot microbiota (Figure 3.3C & D, Excel sheet_WS-2). At the OTU level, bulk soil and rhizosphere bacterial communities share a large degree of similarity. However, when the phylogenetic information was included with OTU relative abundance, a minor separation was observed between the bulk soil and rhizosphere. Whereas, marked segregation was displayed of the root and shoot samples based on both microhabitat zone and tillage effects (R^2 and P values are listed in Table 3.2). These results further support our hypothesis that the WOSR rhizosphere, root and shoot microbiota are colonised by taxonomically distinct communities, which emerge from the soil biota through progressive differentiation and whose composition is modulated, at least in part, by the tillage practices.

Table 3.2 Statistical Analysis of beta Diversity

	Bulk *vs* Rhizosphere		**Root *vs* Shoot**	
	R^2	*P*	R^2	*P*
Bray-Curtis (ADONIS)				
Compartment	0.12218	0.179	0.17214	0.005**
Tillage	0.16541	0.058	0.45064	0.001***
Tillage and compartment	0.06203	0.584	0.12117	0.018*
Weighted Unifrac (ADONIS)				
Compartment	0.20883	0.024*	0.30167	0.001***
Tillage	0.23882	0.012*	0.33495	0.001***
Tillage and compartment	0.09149	0.025*	0.13974	0.003**

Significance levels: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

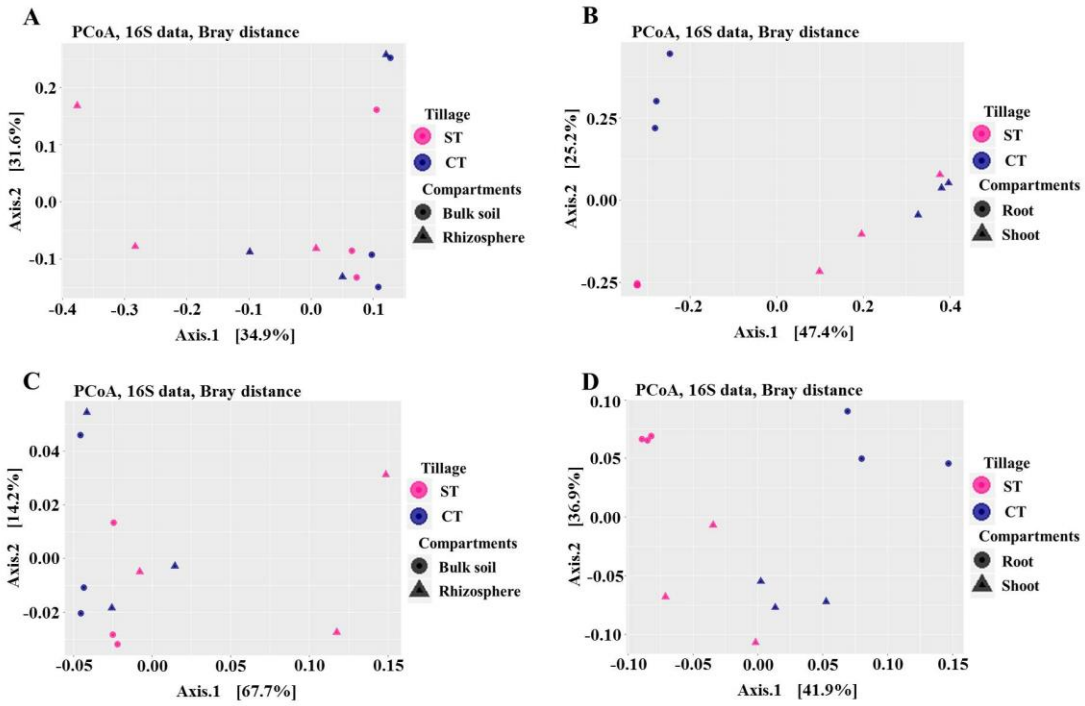


Figure 3.3 Bacterial community structure of bulk soil, rhizosphere, root and shoot under CT and ST tillage practices. Principal Coordinate Analysis (PCoA) based on Bray-Curtis (BC) and Weighted UniFrac (WUF) distances calculated using counts per million transformed OTU abundances. Comparison between the soil samples; bulk soil and rhizosphere (A) BC (C) WUF under CT and ST. Comparison between the plant samples; root and shoot (B) BC (D) WUF under CT and ST. In both panels, colours define the tillage regimes, while shapes depict the indicated compartments. Statistical results of beta diversity are displayed in Excel sheet_WS-2.

3.3.4 Differences in the Microbiota of WOSR microhabitats

To identify OTUs which significantly differentiate the bacterial communities in the four microhabitat zones (bulk soil, rhizosphere, root and shoot) and as a result of the tillage regime (CT and ST), we performed a pair-wise comparison using a model based on a negative binomial distribution. This approach shows that the OTUs identified in the bulk soil are progressively excluded from the rhizosphere (Figure 3.4A & C, Excel sheet_WS-3,4,7,8; Wald-test $P < 0.05$, FDR corrected) and in plant samples, the OTUs found in the roots are gradually excluded from the shoot (Figure 3.4B & D, Excel sheet_WS-5,6,9,10; Wald-test $P < 0.05$, FDR corrected) regardless of the tillage regime. Individual bacterial OTUs were enriched in each microhabitat and contributed to differentiating these communities. Intriguingly, these OTUs represent just a minor fraction of the total WOSR microbiota. For instance, under CT, we observed no significant OTUs enrichment in the rhizosphere compared to bulk soil which suggests that both microhabitat zones share very similar bacterial members. While under ST, 118 and 20 OTUs were enriched in the bulk soil and rhizosphere respectively (Figure 3.4D, Excel sheet_WS-7, 8, Wald test, $P < 0.05$, FDR corrected). Our analysis showed that in rhizosphere soil, there was little or no significant enrichment of OTUs as a consequence of the tillage practices used. Whereas in plant samples, 368 and 39 enriched OTUs differentiated root and shoot microhabitats under CT, respectively (Figure 3.4B, Excel sheet_WS-5,6, Wald test, $P < 0.05$, FDR corrected), and 174 and 51 enriched OTUs under ST respectively (Figure 3.4D, Excel sheet_WS-9, 10, Wald test, $P < 0.05$, FDR corrected). Thus, the significant enrichment of individual members of the plant habitat bacterial communities represents a distinctive feature of the WOSR root and shoot microbiota. This feature displayed a clear microhabitat zone- and tillage-dependency (Figure 3.5). None of the enriched OTUs appeared conserved across the plant microhabitat zones and tillage method and the root and shoot profiles were characterised by distinct patterns. For instance none of the enriched OTUs were shared between the root and shoot in each tillage practice. Moreover, the root profile was characterised with markedly distinct OTU enrichment: 205 OTUs under CT and just 10 under ST.

To further evaluate significantly enriched OTUs as a result of tillage regimes, we performed a similar pair-wise comparison using negative binomial distribution (Figure A3.5, Table A3.3, Wald test, $P < 0.05$, FDR corrected). This approach

showed that in bulk soil, there was no significant difference in the OTU enrichment between both tillage practices. Under CT there were 5, 13 and 9 enriched OTUs identified in the rhizosphere, root and shoot, respectively. Under ST there were 9, 10 and 1 enriched OTUs identified in the rhizosphere, root and shoot, respectively (Table A3.3).

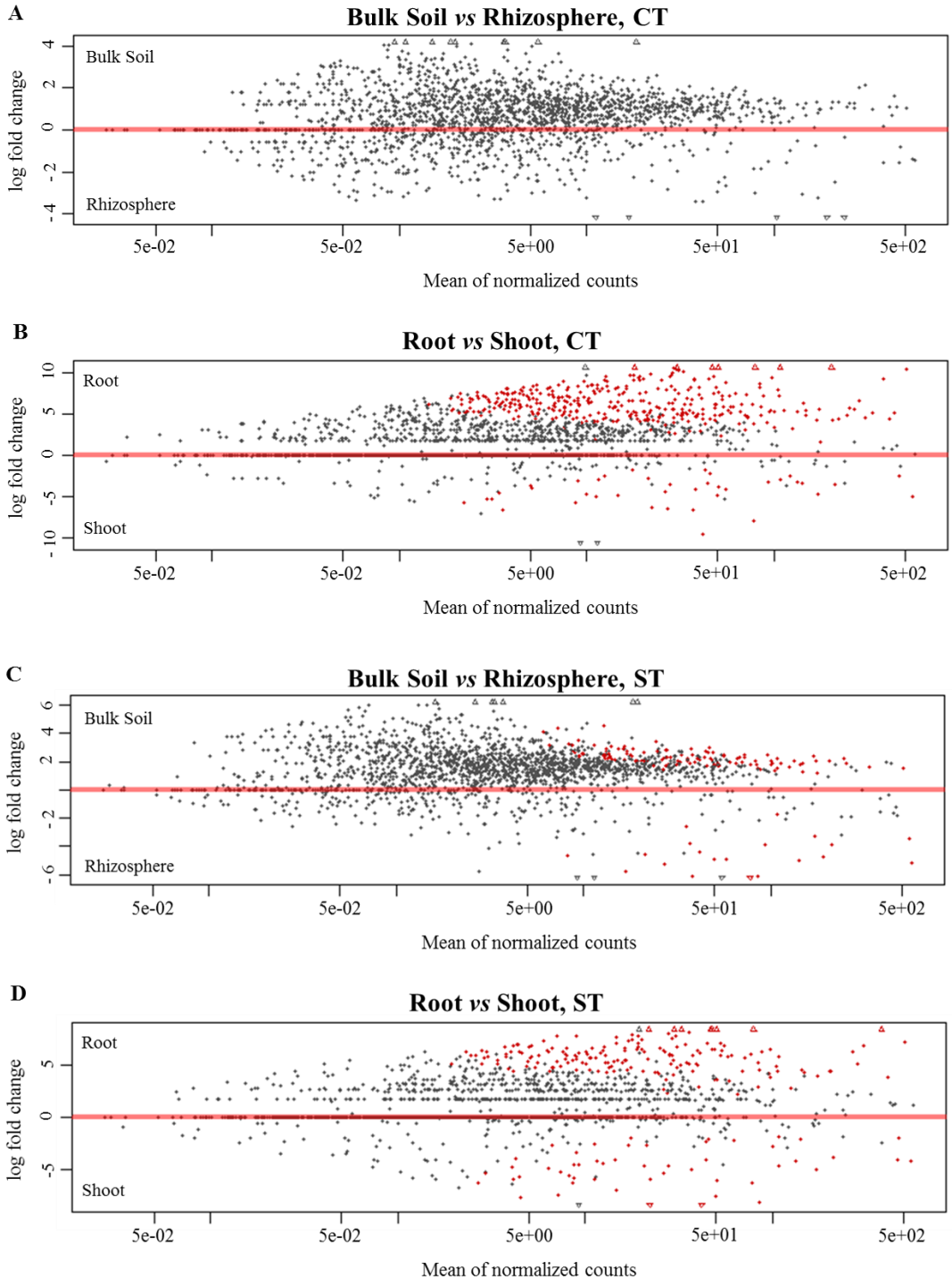


Figure 3.4 Pair-wise comparisons of the compartments under tillage regimes for enriched OTUs. Comparison of bulk soil and rhizosphere under (A) CT (C) ST. Comparison of root and shoot under (B) CT (D) ST. In each plot, the shapes depict individual OTUs whose position on the x-axis reflect their abundance (normalised counts) and the position on the y-axis the fold change in the indicated comparison. The red colour depicts OTUs whose abundance is significantly different in the indicated comparisons (Wald test, $P < 0.05$, FDR corrected). Taxonomy information of significantly enriched OTUs in each compartment under both tillage are displayed in Excel sheet_WS-3 to 10.

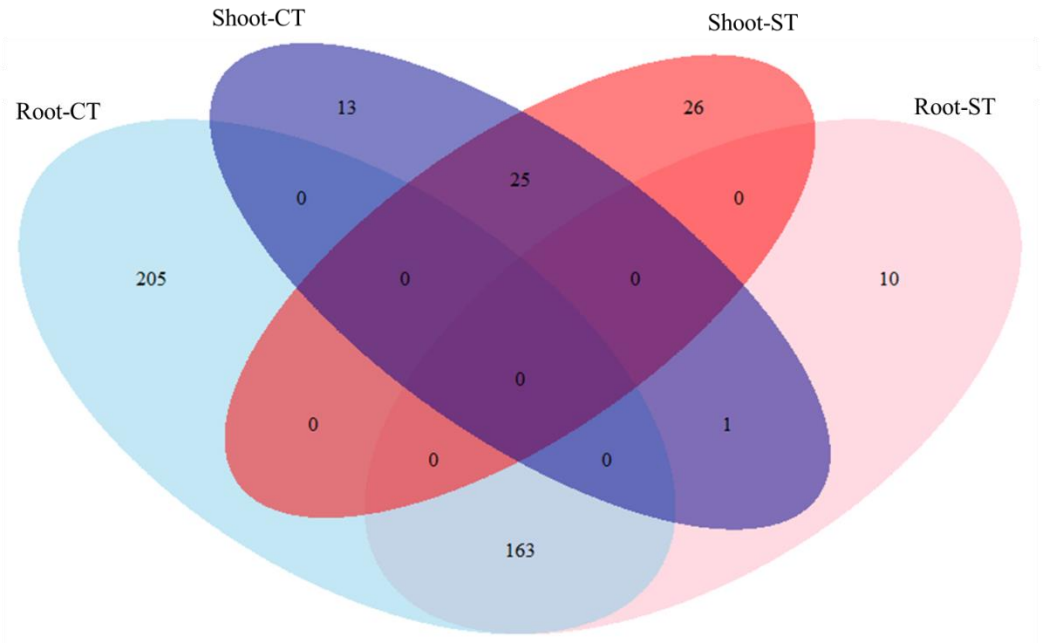


Figure 3.5 Venn diagram displays the number of OTUs that significantly differentiate root and shoot compartments in the indicated tillage regime; CT and ST (Wald test, $P < 0.05$, FDR corrected).

3.4 **Discussion**

This study focused on the effect of tillage practices conventional versus conservational strip tillage, on the microbiota associated with WOSR. The bulk soil bacterial communities under both tillage systems were dominated by Proteobacteria, Actinobacteria, Acidobacteria and Verrucomicrobia phyla. These phyla typically form a major part of the microbial composition of agricultural soils (Fierer & Jackson, 2006, Montecchia *et al.,* 2015). We found no significant difference in number of OTUs between conventionally tilled soils and in strip–tilled soil. This is in contrast to the findings of Smith *et al.* (2016) who found that conventionally tilled fields had more OTUs than conservation tilled fields; they also found that bacterial abundance was very low in conventionally tilled soil. We also did not see any significant difference in the number of OTUs in the rhizosphere between conventionally tilled soil and strip–tilled soil. Although we did observe increases in Bacteroidetes in the rhizosphere under ST, there was no major significant difference between the rhizosphere soils and the bulk soils. This would seem to indicate a very limited 'rhizosphere effect' in WOSR (i.e., an increased abundance, structural enrichment and diversification of the microbial communities inhabiting the rhizosphere compared to bulk soil). This was further supported by our alpha diversity analysis which showed that the microbiota of the bulk soil and rhizosphere were not distinct from each other. This is in striking contrast with other studies reporting a marked structural differentiation of the rhizosphere profiles from the bulk soil of other annual plants, such as the monocotyledons: barley (Bulgarelli *et al.*, 2015), maize (Peiffer *et al.*, 2013) and rice (Edwards *et al.*, 2015) and an earlier study conducted on OSR using low-resolution profiling techniques (Costa *et al.*, 2006). Our observations are similar to the findings of Bulgarelli *et al.* (2012) and Lundberg *et al.* (2012) in *A. thaliana* (which is from the same botanical family as OSR) who reported the resemblance of rhizosphere microbiota to the bacterial community of bulk soil samples in multiple soil types. However, our beta diversity Weighted UniFrac analysis showed minor separation between bulk and rhizosphere bacterial communities based on the tillage effect. Again this is in contrast to previous studies which have observed a much more pronounced effect of conventional and conservational tillage practices on soil microbial communities (Carbonetto *et al.*, 2014, Smith *et al.*, 2016, Degrune *et al.*, 2017).

The bacterial communities associated with the roots and shoots of WOSR were found to be dominated by the bacterial phyla Proteobacteria, Actinobacteria and Bacteroidetes. These three phyla comprised 83-91 % of the root microbiota and 98-99 % of the shoot microbiota. This is similar to what has been reported for *A. thaliana*; (Bulgarelli *et al.*, 2012, Lundberg *et al.*, 2012, Bodenhausen *et al.*, 2013) as well as for other monocotyledon and dicotyledon species (reviewed in Hacquard *et al.* (2015). Alpha and beta diversity analysis showed pronounced differences in the root and shoot microbiota. There was a clear reduction in OTU number, richness and abundance from the rhizosphere into the root and from the root into the shoot. This observation mirrors the multi-step selection processes proposed for the plant microbiota (Bulgarelli *et al.*, 2013, Bulgarelli *et al.*, 2015, Edwards *et al.*, 2015), where a combination of host-microbe and microbe-microbe interactions progressively define the microhabitat zones of the plant microbiota.

When we looked at the effect of tillage practice on the root and shoot microbiota, our alpha diversity analysis suggested that tillage method had little effect on the shoot microbiota. However, alpha diversity was remarkably different in the roots under strip tillage compared with conventional tillage. This observation was further supported by the PCoA plots of beta diversity which showed pronounced separation of both the root and shoot bacteria communities based on tillage practices. This difference is possibly driven by changes in physical properties of soil such as texture, structure, permeability, nutrient content or pH due to the different tillage methods (as the plant genotype was the same in both treatments, and therefore selective pressure from the plant should be the same in both treatments) (Mathew *et al.*, 2012, Smith *et al.*, 2016).

Degrune *et al.* (2017) reported a short term temporal change in soil community structure as a result of tillage practice and reported that these changes became less significant at the later growing stages of the plant. Our results are in agreement with this, where at the harvesting stage of WOSR we observed very similar microbiota profiles of soils subjected to conventional and strip-tillage systems. We hypothesise that different tillage practices cause short term changes in the bulk soil microbiota, and although these changes were not lasting in the bulk soil, they are significant enough to affect the initial colonisation and community structure of the plant at the germination and seedling stages. This in turn leads to significant and lasting effects on the plant microbiota. These observations prompt further

investigation aimed at elucidating the long term impact of tillage practices on the composition of the soil and WOSR microbiota and their ecological services.

Our results showed that the root microbiota appears to be sensitive to tillage practice. This is evidenced by a differential enrichment of individual bacteria likely derived from the soil biota. Are these enriched bacteria a source of plant probiotic functions and what kind of functions can they provide to their host plants? Answering these questions will bring farmers a step closer to rationally manipulate the plant microbiota through soil tillage management.

Chapter 4

Conservation strip tillage leads to permanent alterations in the rhizosphere microbiota of *Brassica napus* crops

This work is submitted to Phytobiome Journal as:

Ridhdhi Rathore, David N. Dowling, Patrick D. Forristal, John Spink, Paul D. Cotter, Kieran Germaine (2018). Conservation strip tillage leads to permanent alterations in the rhizosphere microbiota of *Brassica napus* L. crops. Phytobiome (PBIOMES-0218-0005-R). (under review)

4.1 **Introduction**

Soil is one of the richest, most diverse and wide ranging ecosystems on earth and provides many essential ecosystem services and products such as nutrient cycling, water filtration and a growth medium for food production (Altieri, 1999). The provision of these ecosystem services are mostly driven by the complex interactions of soil biota and abiotic parameters (Kibblewhite *et al.*, 2008). These soil microbes are sensitive and rapid indicators of perturbations and land use changes. Thus, the quantitative description of soil biota structure, composition and diversity is a potential tool for soil quality assessment (Zelles, 1999).

Tillage systems are one of the most significant agricultural management practices which can alter the soil characteristics by changing the soil's physical, chemical and biological properties. These changes ultimately influence soil microorganism diversity, community structure and soil microbial processes (Jangid *et al.*, 2008, Vian *et al.*, 2009). Conventional plough tillage is the most common tillage system utilised by farmers. Conventional tillage refers to the inversion of the soil to a depth of ~25 - 30 cm by mouldboard ploughing. This mechanical disruption of soil may adversely affect long-term soil productivity due to soil compaction, erosion and loss of soil organic matter (Vian *et al.*, 2009). As a result, farmers are showing interest in alternative, conservation tillage systems which minimise the soil disturbance and helps to maintain soil productivity (Lahmar, 2010).

Several studies have reported the effect of agricultural management and tillage practices on soil microbial communities (Mathew *et al.*, 2012, Zhang *et al.*, 2012, Carbonetto *et al.*, 2014, Smith *et al.*, 2016, Degrune *et al.*, 2017, Rathore *et al.*, 2017). However for many crops, including *B. napus*, the impact of tillage practices on the rhizosphere microbiota are not well understood. Rhizosphere microbes are critical in supporting the exchange of resources between the plant and their associated soil environment. Rhizosphere processes contribute to plant health and development, disease suppression and ultimately to crop productivity (Peiffer *et al.*, 2013). Soil microbes are affected by various abiotic factors such as soil texture, moisture, pH, organic matter content, oxygen and nutrient availability, soil aeration, temperature and biotic factors such as plant communities, presence of other soil organisms like fungi and soil fauna (Garbeva *et al.*, 2004, Hery *et al.*, 2007, Xu *et al.*, 2009, Degrune *et al.*, 2017). Many researchers have reported that some of these parameters are altered, to various degrees, by the different tillage practices utilised

by farmers (Six *et al.*, 1999, Lipiec *et al.*, 2006, Vian *et al.*, 2009, Degrune *et al.*, 2017). This disturbance may influence the soil microbial communities and associated ecosystem services.

In addition to tillage practices, plant developmental stages are also an important driver of bacterial community structure and composition (Bulgarelli *et al.*, 2012, Chaparro *et al.*, 2014). The dynamics of bacterial community changes over the life-time of a plant are mainly brought about by root development and associated changes in rhizodeposition (Philippot *et al.*, 2013). Earlier studies have reported the impact of growth stages on soil and rhizosphere microbial community structure and dynamics under field and glasshouse conditions (Shi *et al.*, 2011, Bulgarelli *et al.*, 2012, Zhang *et al.*, 2012). However, these studies have not reported the impact of agricultural practices on the rhizosphere microbiota. Therefore the aim of this study was to explore the niche separation of rhizosphere from the bulk soil under the influence of plant developmental stages and explore the tillage-dependent effects on WOSR rhizosphere microbiota. We hypothesised that WOSR rhizosphere microbiota separation from the bulk soil biota would be influenced by the plant growth stages and soil tillage fine-tunes its composition.

4.2 Materials and methods

4.2.1 Experimental site

The WOSR field experiment for microbiome study was conducted during the year August 2013 to July 2014 at Teagasc Crop Research Centre, Oak Park, Carlow, Ireland 52.86 ^{o}N and 6.92 ^{o}W.

4.2.2 Experimental design

Samples for this study were taken from a field experiment evaluating the effect of crop establishment systems on the growth and development of WOSR. The establishment systems comprised of (1) a conventional tillage (CT) plough based system and (2) a low-disturbance conservation strip tillage (ST) system. The WOSR variety 'Compass' was used. The trial was a randomised block design with three replications where individual plot dimension was 24 m x 4.8 m. For the WOSR rhizosphere microbiota study, the plant and bulk soil samples were taken from these two establishment systems (CT and ST) in three replicated plots. The previous crop was winter barley and cereal crops had been sown for more than 5 years previously in the same field. The detailed experimental design, crop establishment and management practices are provided in section 3.2.1.

4.2.3 Sample collection and DNA extraction

Bulk soil and root samples were collected from the CT and ST treatments, in triplicates from three replicated plots per treatment. The samples were collected at three different plant developmental stages; vegetative stage (~120 days after sowing), flowering stage (~240 days after sowing), and at harvesting (~330 days after sowing) stage. Bulk soil samples were collected from a depth of 0–25 cm, from the edges of each plot, using a hand auger, in triplicate. For each plot, composite soil samples were prepared by thoroughly mixing the soil samples, and a representative subsamples (3 samples/plot) of this was collected in sterile 50 mL Falcon tubes. The excess soil from the roots was removed by manual shaking and collected the soil still attached to the root with sterile forceps in pre-labelled sterile 50 mL falcon tubes. All the samples were stored in -80 ^{o}C until required for the DNA extraction.

DNA extractions were performed on 3 bulk soil and 3 rhizosphere soil samples per plot with 3 replicated plots per treatment (CT and ST). For DNA extraction, 0.25 g of soil was taken individually from each composite soil sample and processed according to the protocol from MoBio PowerSoil^{TM} DNA isolation kit (Carlsbad, CA, United States). Total soil DNA was eluted in 50 µL of sterile water (Sigma–Aldrich). Concentration and purity of DNA was determined by Qubit 4 fluorimeter and Nanodrop spectrophotometry (Thermo Scientific, Wilmington, DE, United States) respectively. Post quantification, all DNA samples were normalized to10 ng μL^{-1}. The three DNA samples from each microhabitat zone per block were pooled (e.g. the three DNA samples of rhizosphere soil samples from block 1 were pooled) to give representative DNA samples of bulk soil and rhizosphere from each block.

4.2.4 Library preparation of 16S rRNA gene amplicon

The amplicon library of bacterial DNA was generated using the PCR primers: 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWG CAG-3′), 785R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACT ACHVGGGTATCTAATCC-3′), with Illumina adapter overhang sequences (underlined) which covered ∼464 bp of the hypervariable regions V3 and V4 of the 16S rRNA gene (Klindworth et al., 2013). Amplicons were generated, purified, indexed and sequenced with some modifications according to the Illumina MiSeq 16S Metagenomics Sequence Library Preparation protocol (16S-Metagenomic-library-prep, 2014). An initial PCR reaction contained 25 µL of 2 x KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, Wilmington, MA, United States), 1 µL of each forward and reverse primer (1 µM), 2.5 µL of DNA (∼10 ng μL^{-1}) and 20.5 µL of nuclease free H_2O in a total volume of 50 µL. The PCR reaction was performed on a 96-well Thermocycler using the following program: 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension step at 72 °C for 5 min. All amplicons were cleaned using Ampure DNA capture beads (Agencourt-Beckman Coulter; Inc.) following addition of Illumina sequencing adapters and dual-index barcodes to each amplicon with the Nextera-XT Index kit (Illumina Inc., San Diego, CA, United States) according to the manufacturer's instructions. The amplicon libraries were pooled in equimolar concentrations. The final library was paired-end sequenced at 2 × 300 bp using a MiSeq Reagent Kit v3

on the Illumina MiSeq platform. Sequencing was performed on the Next Generation Sequencing Platform at Teagasc Moorepark research centre, Fermoy, Cork, Republic of Ireland.

4.2.5 Amplicon data analysis

16S rRNA gene sequences were analyzed using Usearch v9.2.64 32 bit (Edgar, 2013) and Qiime, v1.9.0 (Caporaso *et al.*, 2010), unless otherwise specified the default parameters were used. Paired-end reads were merged using the command fastq mergepairs in Usearch by specifying a minimum overlap of 16 bp. Barcode sequences were removed from the merged paired-end sequences using the command extract_barcodes.py in Qiime. We used Usearch to demultiplex the pre-processed sequencing reads and to generate a quality report. We used the fastq_filter function in Userach to truncate all the reads to a length of 400 bp and discard sequences shorter than this length and sequences that contained more than four expected continuous base errors per read. The retained high-quality sequencing reads then clustered into operational taxonomic units at 97 % sequence identity using the Userach pipeline. Singletons were discarded from further analysis and the "Gold" reference database was used to identify and remove chimeras. Taxonomic classification of OTU-representative sequences was performed in Qiime using RDP classifier (Wang *et al.*, 2007) trained against the Greengenes database (DeSantis *et al.*, 2006), release 13_5. Likewise, we used OTU representative sequences to generate a phylogenetic tree in Qiime using 'Phynast' as alignment method. The generated OTU table, taxonomy information and phylogenetic tree were used to implement the ecological and statistical analyses.

4.2.6 Statistical analysis

Data analysis and visualization were performed using Phyloseq (McMurdie & Holmes, 2013) package from R operated through R Studio v0.99.893. All OTUs belonging to chloroplast and mitochondria were identified and removed from the data set prior the analysis. For alpha diversity analysis, observed OTUs, Chao1 and Shannon indexes, normal distribution of the data were checked with the Shapiro-Wilk test. Significant differences in the variance of parameters were evaluated,

depending on the distribution of the estimated parameters, with analysis of variance (ANOVA) to identify significant differences between the plant developmental stages, two tillage systems and microhabitat zones. *Post hoc* comparisons were conducted using the Tukey test. For such analysis, sequencing reads of soil samples were rarefied at an even sequencing depth of 5,025 reads per sample. To compare community diversity between the samples (beta-diversity), Principal Coordinate Analysis based on Bray-Curtis and Weighted UniFrac distances were calculated by using counts per million transformed OTU abundances. Permutational multivariate analysis of variance using distance matrices was performed in R using the -adonis function to define the strength and significance of tillage and/or microhabitat zone in determining variation of microbial abundances over the growth stages of WOSR. A differential analysis of the OTUs relative abundances using moderated shrinkage estimation for dispersions and fold changes as an input for a pair-wise Wald test was carried out in DESeq2 package from R v1.14.1 (Love *et al.*, 2014). This test identifies the number of OTUs significantly enriched at the different developmental stages corrected for the tillage practices in rhizosphere and bulk soil, tillage practices corrected for individual growth stage of each microhabitat zone, microhabitat zones corrected for the growth stages in CT and ST with an adjusted *P* value (False Discovery Rate, FDR $P < 0.05$). ANOVA analysis was carried out to see the effect of plant developmental stages, tillage and microhabitat zones on the top nine most abundant bacterial phyla. Relative abundance was calculated on the rarefied dataset for such analysis. For bacterial taxonomic classification at phylum and family level, the abundance data were transformed in percentage to calculate the percent relative abundance. In addition, ANOVA analysis was calculated of normally distributed bacterial sequence reads at phylum level, to see the effect of plant developmental stages, tillage practices and microhabitat zones of the top nine most abundant bacterial phyla (with an alpha level of 0.05). Phylum level abundance was calculated based on the number of sequence reads classified to phylum level.

4.3 **Results**

The influence of tillage practices, (CT and ST) on the bacterial communities in the bulk soil and rhizosphere over three growth stages of WOSR was analysed by amplicon (16S rRNA) next generation sequencing Illumina MiSeq platform. The analysis generated 1,823,764 sequence reads of which 1,524,423 (~86.1 % total sequence reads) were retained upon quality trimming, 1,400,091 were retained after removing de-replicate sequence reads and 1,049,731 sequence reads were retained upon singletons removal. These 1,049,731 sequence reads were clustered into 3,755 OTUs at >97 % sequence similarity. After removal of chimeric and a small fraction of plant derived (chloroplast/mitochondria) OTUs, 3,594 OTUs were used for the downstream analysis.

Our analysis showed that there were 386 OTUs shared among the 18 rhizosphere samples analysed in our study. OTU1 (f_*Bradyrhizobium*) was the only OTU that was consistently found in all 18 rhizosphere samples, in abundances of greater than 1% of the totals reads per sample. It had an average abundance of 3.6% of the total reads/sample. Other OTUs that were strongly associated with the rhizosphere but only found in 13-17 out of the 18 samples included OTU5 (a member of the phylum Chloroflexi) with an average abundance of 2.8% per sample, OTU3 (f_*Micrococcaceae*) with an average abundance of 2.7% per sample, OTU33 (g_*Mycobacterium*) at 1.6%, OTU29 (f_*Sphingomonadaceae*) 1.7%, OTU8 (g_*Candidatus*) with an average of 2.2%, OTU7 and OTU4 (both f_*Hyphomicrobiaceae*) at 1.2% and 1.5%, respectively. Collectively these 8 OTUs made up 16.98 $\pm$ 4.1% of the total reads per sample. There were 582 OTUs shared among all of the 18 samples of bulk soil included in our study. OTU1 (f_*Bradyrhizobiaceae*, 3.5%), was the most abundant OTU consistently found in all bulk soil samples. OTU3 (f_*Micrococcaceae*, 1.6), OTU5 (a member of the phylum *Chloroflexi*, 2.7%) and OTU8 (g_*Candidatus*, 2.1%) were consistently found in all bulk soil samples. There was no significant difference in the number of OTUs found in the bulk soil compared to the rhizosphere soil under CT (P = 0.165). However, under ST there was a significantly greater number of OTUs in the bulk soil than in the rhizosphere soil (P =0.021).

4.3.1 Alpha and beta diversities of WOSR microbiota

The alpha diversity within the bacterial communities in both bulk soil and rhizosphere soil at the vegetative, flowering and harvesting growth stages were determined using the observed OTUs, Chao1 and Shannon diversity indices. In addition to this, we examined the effect of tillage practice (CT *vs* ST) on alpha diversity within these samples (Figure 4.1-A, B, C; Excel sheet_WS-1 & 2). The dataset was rarefied to 5,025 sequence reads per sample before calculating diversity indices. In the rhizosphere, there was an increase in alpha diversity at the flowering stage, compared to the vegetative stage, but this diversity declined significantly in the harvesting stage. The alpha diversity of the rhizosphere was higher in the ST system compared to the CT tillage system only at the vegetative stage (Figure 4.1-C). Overall, the alpha diversity in the bulk soil decreases over the life-time of the crop. Alpha diversity in the bulk soil was very similar under CT and ST tillage systems in both the vegetative and harvesting stages, but was much greater in the ST tillage system at the flowering stage. Observed OTUs indices showed a statistically significant difference in alpha diversity as a result of plant growth stages ($P < 0.01$) and microhabitat zones ($P < 0.01$). The Chao1 index also showed a statistically significant due to growth stages ($P < 0.05$), but did not show a significant difference as a result of tillage practices or in the interactions of tillage, growth stages and microhabitat zones ($P > 0.05$). Similarly, the Shannon index showed a statistically significant difference in the diversity due to the plant growth stage, the microhabitat (rhizosphere vs bulk soil) and the interaction of tillage*microhabitat zones ($P < 0.05$), but again did not show a significant difference due to tillage practices.

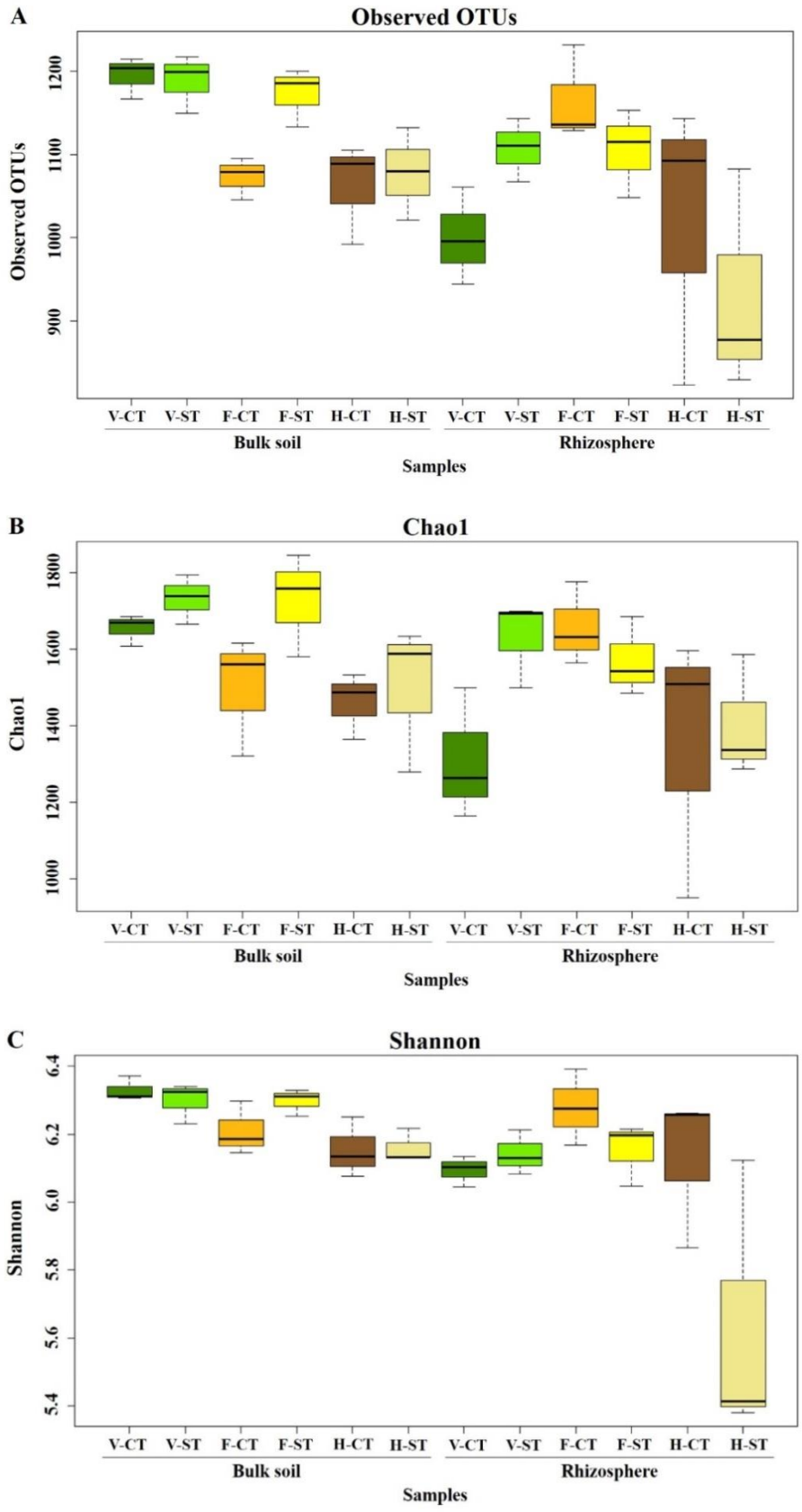


Figure 4.1 Alpha diversity estimates of the bacterial communities associated with rhizosphere and bulk soil under two tillage practices; CT and ST at three plant

growth stages (V-vegetative, F-flowering & H-harvesting). The alpha diversity estimates; Total number of observed OTUs, Chao1 estimator and Shannon's diversity are displayed in A, B and C respectively. Tillage practices represented by conventional tillage (CT) and conservation strip tillage (ST). Sequencing reads of soil samples were rarefied at an even sequencing depth 5,025 reads/sample prior the analysis. Statistical analysis of alpha diversity is displayed in Excel sheet_WS- 1 & 2. Data are represented by three replicates of each sample. Centre line of boxes represent median of samples. The upper and lower sides of the boxes represent the third and first quartiles, respectively. Whiskers represent ±1.5 times the interquartile range.

To visualize whether tillage regimes, growth stages and microhabitats were important factors driving microbial beta diversity, Bray-Curtis and Weighted UniFrac dissimilarity matrices were used with Principal Coordinate Analysis (Figure 4.2). At vegetative stage, PCoA analysis showed a clear separation between the two tillage practices in both BC ($P < 0.05$) and WUF ($P < 0.01$) matrices. In BC matrix, bulk soil and rhizosphere microhabitats under CT also presented minor separation though it was not significant ($P = 0.057$; Figure 4.2A) whereas WUF matrix exhibited the clear clustering of bulk soil samples under CT (Figure 4.2B). Flowering stage showed no influence of tillage as well as microhabitat type on soil microbiota structure in both dissimilarity matrices ($P > 0.05$; Figure 4.2C & D). Whereas at harvesting stage, rhizosphere microbiota under ST presented the strong clustering compared to other soil samples and showed the significant influence of tillage and microhabitat type in both BC ($P < 0.02$) and WUF ($P < 0.01$) matrices. R^2 and P values of ADONIS based Bray-Curtis and Weighted Unifrac distance matrices are listed in Table 4.1.

Table 4.1 Statistical analysis of beta diversity

Vegetative stage				
	Bray-Curtis		**Weighted UniFrac**	
	R^2	***P***	**R^2**	***P***
Tillage	**0.172**	**0.037***	**0.207**	**0.011***
Microhabitat zones	0.149	0.057	0.142	0.059
Tillage * Microhabitat zones	0.069	0.498	0.060	0.559
Flowering stage				
	Bray-Curtis		**Weighted UniFrac**	
	R^2	***P***	**R^2**	***P***
Tillage	0.079	0.570	0.060	0.746
Microhabitat zones	0.057	0.804	0.082	0.568
Tillage * Microhabitat zones	0.042	0.956	0.049	0.863
Harvesting stage				
	Bray-Curtis		**Weighted UniFrac**	
	R^2	***P***	**R^2**	***P***
Tillage	**0.214**	**0.023***	**0.215**	**0.014***
Microhabitat zones	**0.206**	**0.021***	**0.215**	**0.016***
Tillage * Microhabitat zones	0.062	0.618	0.084	0.323

Significance levels: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

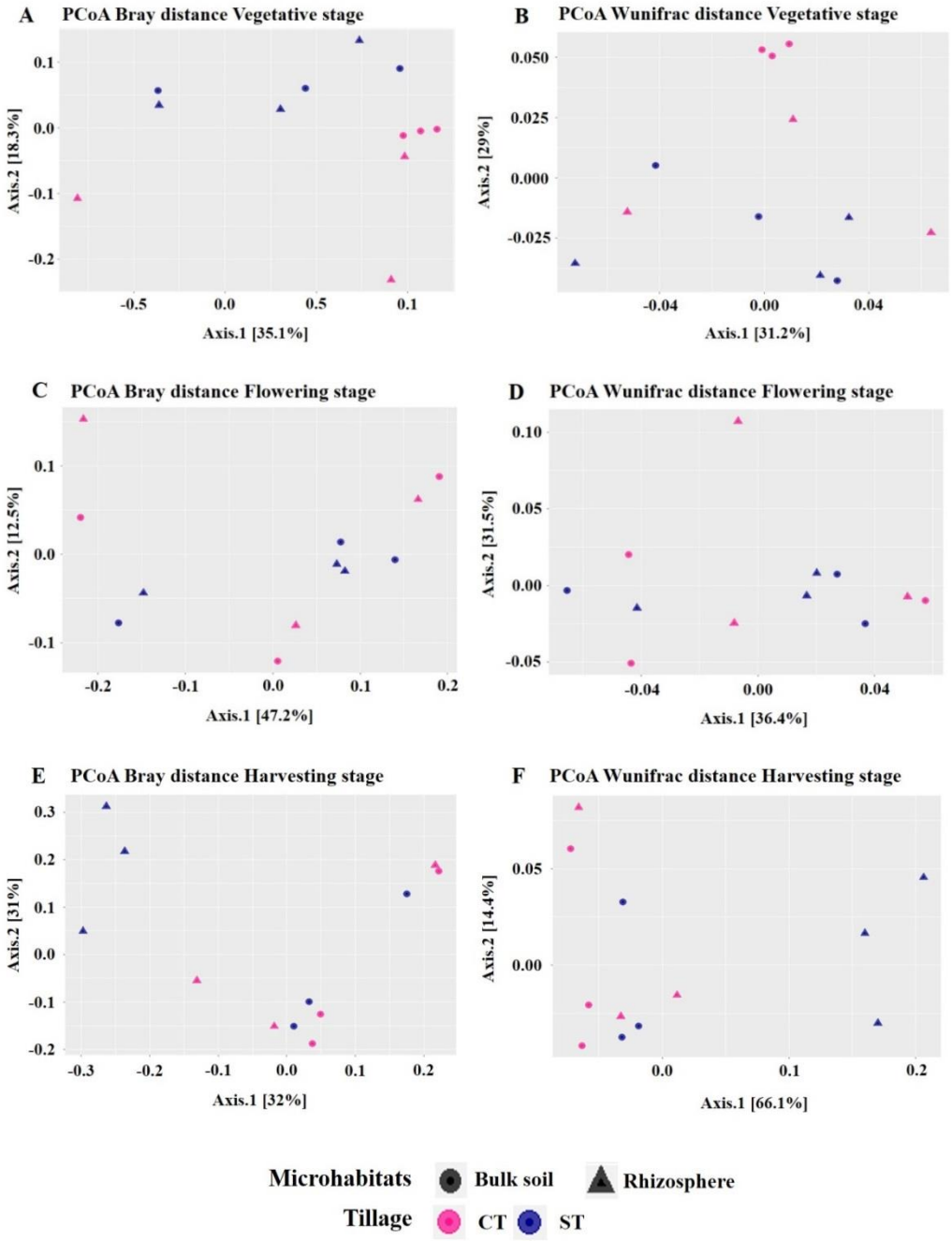


Figure 4.2 Beta diversity estimates of the bacterial communities associated with rhizosphere and bulk soil under two tillage practices; CT and ST at three plant growth stages; vegetative, flowering & harvesting stage. PCoA based on BC and WUF distances calculated using counts per million transformed OTU abundances. Comparison between the bulk soil and rhizosphere at vegetative stage (A) BC (B) WUF, flowering stage (C) BC (D) WUF, and harvesting stage (E) BC (F) WUF under CT and ST regimes. In both panels, colours define the tillage practices, while shapes depict the indicated microhabitats. Statistical results of beta diversity are displayed in Excel sheet_WS-3.

4.3.2 Drivers of WOSR microbiota alterations

To identify OTUs that were responsible for significant differences in the communities with respect to plant growth stages, tillage practices and microhabitat zones, we performed a pair-wise comparison using a model based on a negative binomial distribution (Table 4.2, Excel sheet_WS-4, 5, 6 & 7; Wald-test $P < 0.05$, FDR corrected). Here, individual OTUs are identified which differentiate the growth stages, tillage practices and the microhabitat zones (Table 4.2A, B & C respectively). OTUs differentially enriched in the indicated pair-wise comparison are defined by the mean relative abundance and the logarithmic fold change in abundance. In all comparisons a positive fold change is associated with the enrichment of the OTU in a specific sample type.

To investigate the impact of growth stages on WOSR soil microbiota, the OTUs identified in each growth stage were progressively excluded from the other two growth stages. For example, OTUs identified in vegetative stage were excluded from the flowering stage (vegetative *vs* flowering), and harvesting stage (vegetative *vs* harvesting). Individual bacterial OTUs which were enriched or depleted in each growth stage contributed to differentiating the bacterial communities. Intriguingly, these OTUs represented just a minor fraction of the total WOSR microbiota. The results showed that at different time points in the rhizosphere under CT, very few OTUs were enriched (Table 4.2A, Excel sheet_WS-4). Likewise in bulk soil under both tillage practices, at different growth stages, few OTUs were significantly enriched, suggesting a relatively stable bacterial community (Table 4.2A, Excel sheet_WS-5, $P < 0.05$, FDR corrected). Furthermore, we observed no significant OTU enrichment in rhizosphere under ST at the vegetative and flowering stage. However, when we compared the flowering stage with the harvesting stage we observed 60 OTUs enriched in the flowering stage and 11 OTUs enriched in the harvesting stage. Likewise when we compared the harvesting stage with the vegetative stage we observed 5 OTUs enriched in the harvesting stage and 57 OTUs enriched in the vegetative stage (Table 4.2A, Excel sheet_WS-4, $P < 0.05$, FDR corrected).

While tillage effect was studied using the same negative binomial distribution model with Wald test ($P < 0.05$, FDR corrected), we observed that, in rhizosphere, only harvesting stage showed significant OTUs enrichment between CT and ST practices (22 OTUs in CT and 4 OTUs in ST; Table 4.2B, Excel sheet_WS-6; $P <$

0.05, FDR corrected). Additionally, bulk soil did not show any significant OTUs enrichment in tillage practices at any plant growth stage.

With the similar approach, when the microhabitats effect was studied on WOSR microbiota, we noticed that under CT, there was no significant OTUs enrichment between the bulk soil and rhizosphere at different growth stages. Whereas under ST, only at harvesting stage, significant OTUs enrichment was observed in bulk soil (31 OTUs) and 1 enriched OTUs in rhizosphere (Table 4.2C, Excel sheet_WS-7; $P < 0.05$, FDR corrected).

Table 4.2 Enriched OTUs retrieved from moderated estimation of fold change and pair-wise Wald test (FDR, $P < 0.05$) of bulk soil and WOSR rhizosphere prokaryotic microbiota profiles, at plant developmental stages, under tillage regimes.

A. Plant growth stage effect (vegetative *vs* flowering *vs* harvesting)			
Comparisons	**Enriched OTUs**	**Conventional tillage (CT)**	**Conservation tillage (ST)**
Rhizosphere			
Vegetative *vs*	Vegetative enriched	1	0
Flowering	Flowering enriched	0	0
Flowering *vs*	Flowering enriched	3	60
Harvesting	Harvesting enriched	1	11
Harvesting *vs*	Harvesting enriched	0	5
Vegetative	Vegetative enriched	1	57
Bulk soil			
Vegetative *vs*	Vegetative enriched	1	0
Flowering	Flowering enriched	0	0
Flowering *vs*	Flowering enriched	1	3
Harvesting	Harvesting enriched	0	1
Harvesting *vs*	Harvesting enriched	4	0
Vegetative	Vegetative enriched	4	0

B. Tillage effect (CT *vs* ST)		
Time-Points	**Enriched OTUs in CT**	**Enriched OTUs in ST**
Rhizosphere		
Vegetative stage (VCT *vs* VST)	0	0
Flowering stage (FCT *vs* FST)	0	0
Harvesting stage (HCT *vs* HST)	22	4
Bulk soil		
Vegetative stage (VCT *vs* VST)	0	1
Flowering stage (FCT *vs* FST)	0	0
Harvesting stage (HCT *vs* HST)	0	0
C. Microhabitat zone effect (Bulk soil *vs* Rhizosphere)		
Time-Points	**Enriched OTUs in Bulk soil (Bk)**	**Enriched OTUs in Rhizosphere (Rz)**
Conventional tillage (CT)		
Vegetative stage (V-Bk *vs* V-Rz)	0	0
Flowering stage (F-Bk *vs* F-Rz)	0	0
Harvesting stage (H-Bk *vs* H-Rz)	1	0
Conservation tillage (ST)		
Vegetative stage (V-Bk *vs* V-Rz)	0	0
Flowering stage (F-Bk *vs* F-Rz)	0	0
Harvesting stage (F-Bk *vs* F-Rz)	31	1

4.3.3 Taxonomic characterisation of bacterial microbiota

After aligning the OTUs with RDP against the Greengenes database, the WOSR soil microbial community was classified into the top ten most abundant bacterial phyla, which accounted for ~97 % of WOSR rhizosphere and bulk soil bacterial community (Figure 4.3; Excel sheet_WS-8). Among them were the phyla Actinobacteria, Proteobacteria, Bacteroidetes and Chloroflexi, which occupied ~60 % of WOSR soil microbiota. To elucidate whether these bacterial phyla showed any significant difference between the developmental growth stages, tillage regimes, and microhabitat zones, ANOVA analysis was carried out on relative abundance of rarefied dataset (Figure 4.4; Excel sheet_WS-9; $P < 0.05$). The phyla Proteobacteria ($P < 0.001$), Chloroflexi ($P < 0.01$) and Firmicutes ($P < 0.05$) showed significant difference in growth stages of WOSR microbiota (Figure 4.4A, B & C respectively). The bacterial abundance of phylum Proteobacteria and Chloroflexi was very similar in all samples at vegetative and flowering stages. However, at harvesting stage the phylum Proteobacteria abundance was remarkably higher while, the abundance of phylum Chloroflexi was lower, especially in rhizosphere under ST. The Firmicutes population showed a significant difference as a result of tillage method ($P < 0.05$) and growth stages. Overall Firmicutes abundance was higher in ST compared to CT, and at harvesting stage compared to other growth stages in both microhabitat zones.

The phylum Actinobacteria displayed a significant difference in tillage*growth stage interaction ($P < 0.05$, Figure 4.4D). The soil samples of ST at harvesting stage showed significantly lower Actinobacteria abundance. While there was a significant difference in the Planctomycetes populations as a result of tillage practice ($P < 0.001$) and a minor contribution of the growth stages and microhabitat zones ($P < 0.01$, Figure 4.4E). Bacterial abundance was significantly higher in CT compared to ST at all growth stages. The bulk soil of CT at vegetative stage and under ST at harvesting stage displayed higher bacterial abundance compared to rhizosphere. Moreover, the bulk soil of CT at the vegetative stage showed the highest abundance of Planctomycetes while the rhizosphere of ST at harvesting stage showed the lowest abundance. A significant difference in the abundance of phylum Verrucomicrobia was observed between the tillage and tillage*microhabitat zones ($P < 0.05$, Figure 4.4F). The bacterial abundance in both microhabitat zones was higher in ST compared to CT at vegetative and flowering stage. However, at harvesting

stage in the rhizosphere, the Verrucomicrobia abundance was lower in ST compared to CT.

Significant differences between the microhabitat zones were observed in abundance of phyla Bacteroidetes ($P < 0.01$) and Acidobacteria ($P < 0.001$). Bacteroidetes abundance in rhizosphere was higher compared to bulk soil, while the rhizosphere of ST at harvesting stage displayed the highest bacterial abundance (Figure 4.4G). Abundance of Acidobacteria was higher in bulk soil compared to rhizosphere under both tillage practices at all growth stages (Figure 4.4H). The phylum Gemmatimonadetes exhibited the significant difference between the microhabitat zones ($P < 0.001$), tillage ($P < 0.05$) and time point ($P < 0.05$). The bacterial abundance was higher in bulk soil compared to rhizosphere under both tillage regimes and at all growth stages except the vegetative stage of ST and flowering stage of CT where the abundance was similar (Figure 4.4I).

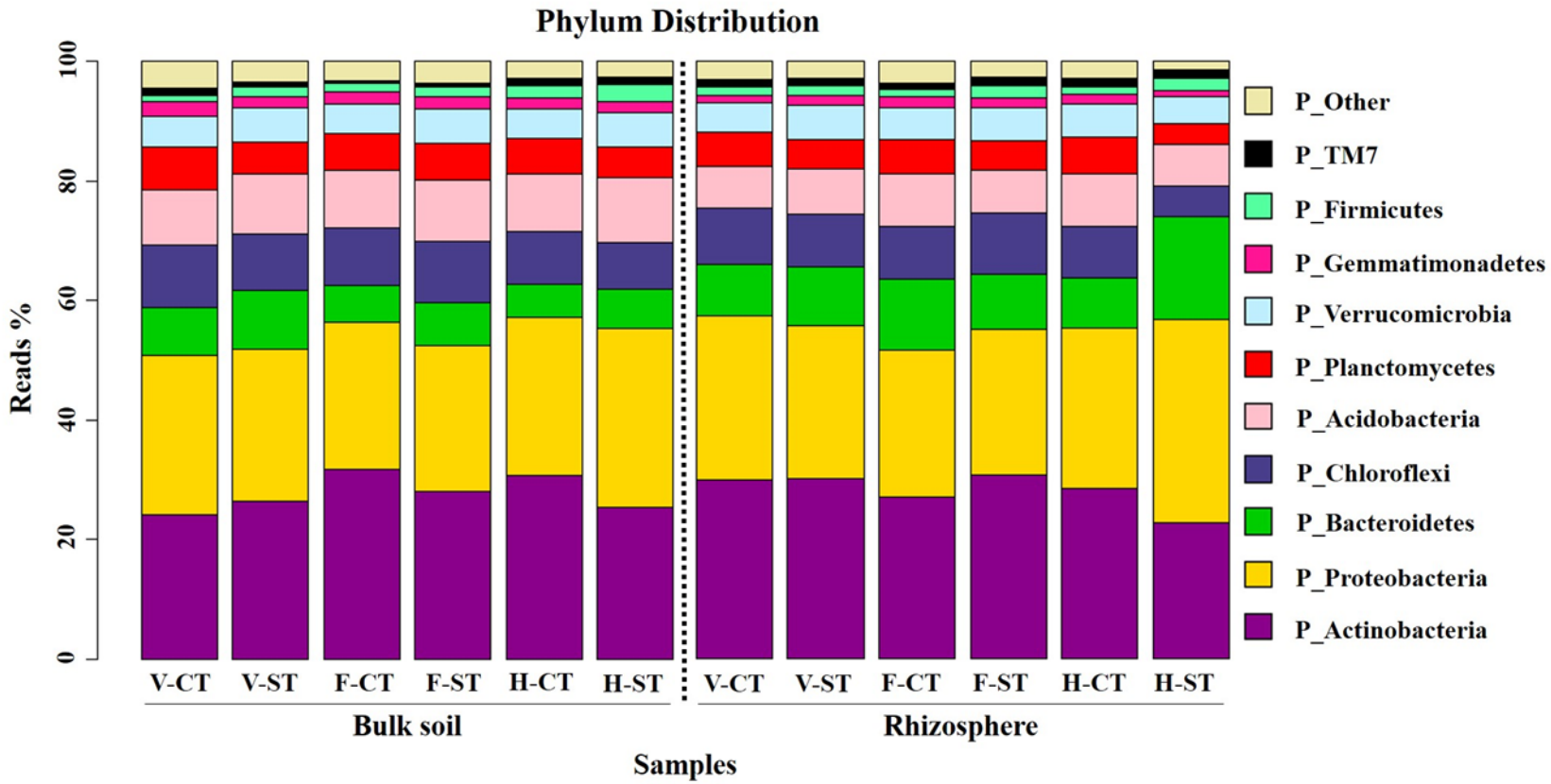


Figure 4.3 Phylum-level relative abundance of bacterial communities across growth stages, tillage practices and microhabitats. Average relative abundance (% of sequence reads) of top ten most abundant prokaryotic phyla associated with bulk soil and rhizosphere of WOSR under tillage practices conventional (CT) & conservation strip (ST) tillage at three different growth stages; V-vegetative, F-flowering and H-harvesting, are displayed in different colours. For each sample type, the number of replicates are n = 3.

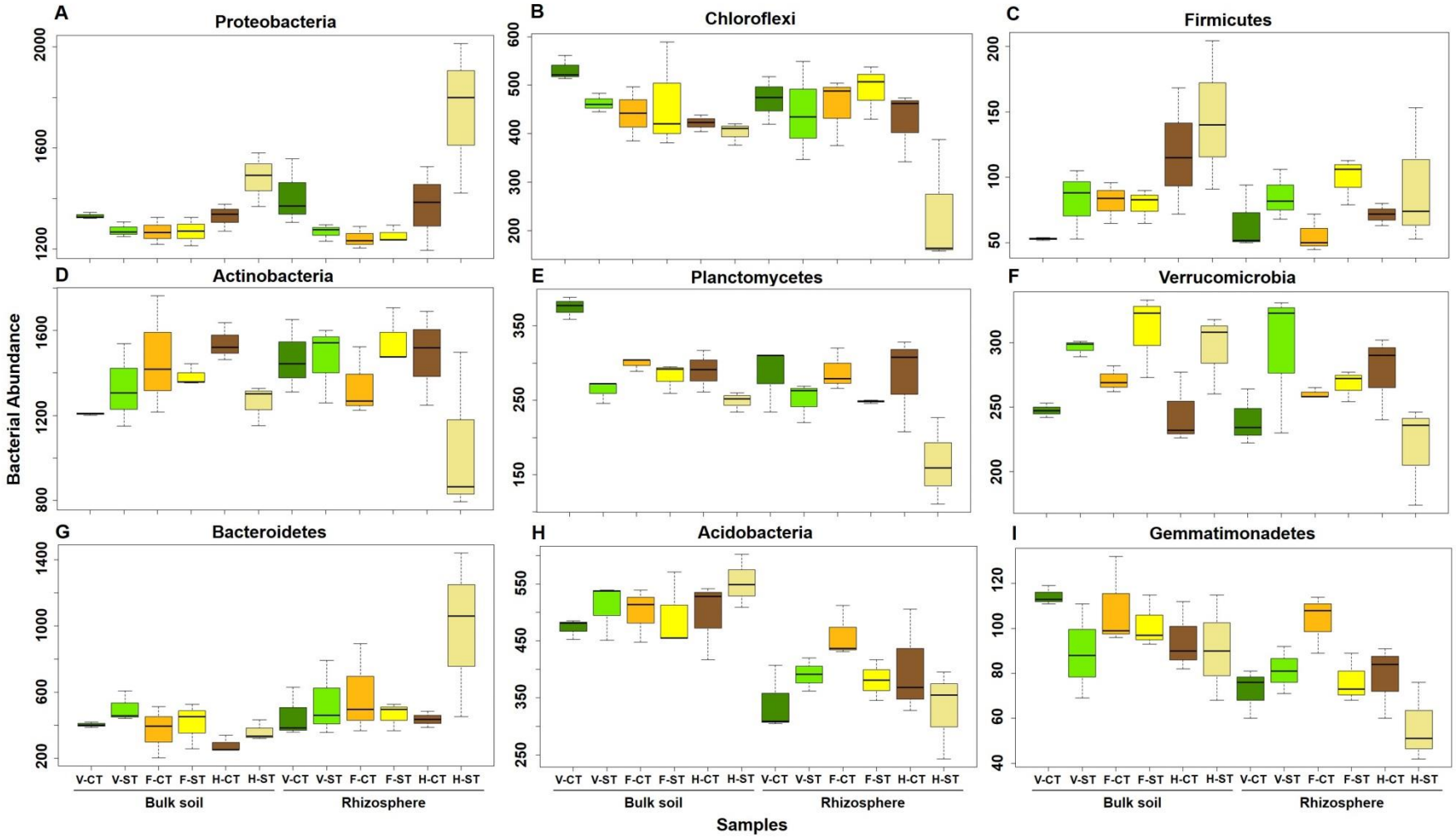


Figure 4.4 Relative abundance of bacterial phylum that significantly (ANOVA; $P < 0.05$) change with growth stages (A, B and C), tillage practices (D, E and F) and microhabitat zones (G, H and I) in rhizosphere and bulk soil under tillage practices; conventional (CT)

and conservation strip (ST) tillage at different plant growth stages. The colours of the box plots represent the three growth stages of WOSR; green (V-vegetative), yellow (F-flowering) and brown (H-harvesting). Dark colours represent the bulk soil and light colours signify rhizosphere. Data are represented by three replicates of each sample. Centre line of boxes represents median of samples. The upper and lower sides of the boxes represent the third and first quartiles, respectively. Whiskers represent ±1.5 times the interquartile range.

We further analysed the soil microbial communities at the family level to determine which families were affected by the tillage practices over the developmental stages, in both bulk soil and rhizosphere (Figure 4.5, Excel sheet_WS-10). The bacterial composition at family level showed that, in the bulk soil ~74.46 % and in rhizosphere ~68.68 % of the OTUs had less than 1 % relative abundance. Additionally, in both microhabitat zones CT samples presented higher number of OTUs that represent less than 1 % relative abundance at family level. Moreover, harvesting stage and especially the ST samples showed higher relative abundance (>1 %) of bacterial composition at family level (Excel sheet_WS-10). We noticed that bacterial diversity between the CT and ST was similar at different growth stages in each microhabitat zone however the abundance of those microbial members was different in CT and ST. For example, the relative abundance of families *Bradyrhizobiaceae, Hyphomicrobiaceae* and *Nocardiaceae* were higher in CT whereas, *Micrococcaceae, Sphingomonadaceae* were higher in the ST rhizosphere samples besides these microbial members present in both CT and ST samples. The abundance of families *Pseudomonadaceae* and *Sphingobacteriaceae* were markedly higher in the ST rhizosphere compared to the CT in rhizosphere samples.

While comparing the bulk soil and rhizosphere at vegetative stage, bacterial family *Cathoniobacteraceae* was present in bulk soil but not in rhizosphere whereas, bacterial families *Nocardioidaceae, Nakamurellaceae* and *Intrasporangiaceae* were observed in rhizosphere, but not in bulk soil. Likewise at flowering stage, *Nakamurellaceae* and *Gaiellaceae* bacterial members differentiated the rhizosphere from the bulk soil. Whereas at harvesting stage, bacterial families such as *Flavobacteriaceae, Intrasporangiaceae, Pseudomonadaceae, Rhizobiaceae, Sphingobacteriaceae, Microbacteriaceae, Nocardioidaceae,* and *Microbacteriaceae* remarkably differentiate the rhizosphere microbiota from the bulk soil biota.

Additionally, at different plant growth stages, the bacterial families *Streptomycetaceae, Rhizobiaceae, Microbacteriaceae, Nocardiaceae,* and *Pseudomonadaceae* only observed at harvesting and *Gaiellaceae* at flowering stage in rhizosphere, whereas *Streptomycetaceae* and *Gaiellaceae* only present at harvesting and vegetative stage respectively in bulk soil. While, some bacterial families such as *Sphingobacteriaceae, Rhizobiaceae, Pseudomonadaceae* and *Microbacteriaceae* were only presented at the harvesting stage in rhizosphere and

were not present in bulk soil. The bacterial family *Nakamurellaceae* was observed at vegetative and flowering stage but not at harvesting stage. On the other hand, the family *Intrasporangiaceae* was observed at vegetative stage, it was not observed at the flowering stage, and then reappeared at harvesting stage in the rhizosphere. Both of these families were not observed in bulk soil. In the rhizosphere, *Sphingomonadaceae* and *Micrococcaceae* abundances were dramatically increased at harvesting stage compared to vegetative and flowering stage.

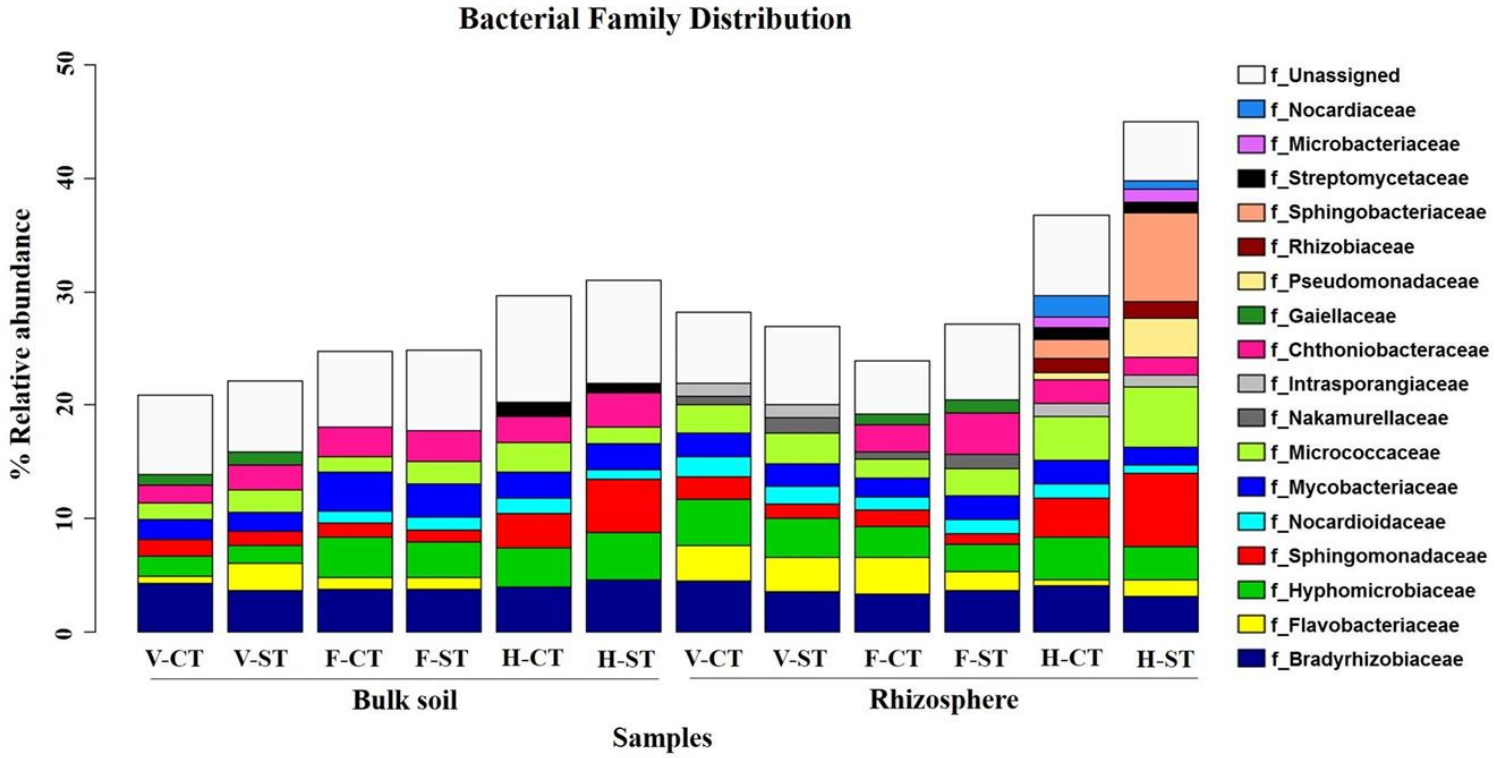


Figure 4.5 Family-level relative abundance of bacterial communities across growth stages, tillage practices and microhabitats. The bacterial families that represent only >1 % average relative abundance of sequence reads in bulk soil and rhizosphere of WOSR under conventional tillage (CT) and conservation strip tillage (ST) practices at three plant growth stages; vegetative (V), flowering (F), harvesting (H) are displayed in this figure in different colours. The sequencing reads of samples were rarefied at an even sequencing depth 5,025 reads/sample prior to analysis.

4.4 **Discussion**

We investigated the effects of tillage and plant growth stage on the rhizosphere microbial community in WOSR, in comparison to the bulk soil bacterial community under either CT or ST practices. This analyses involved the extraction of DNA from soil and rhizosphere samples, amplification of a 400 bp fragment of the 16S rRNA gene, subsequence next generation sequencing of these fragment and bioinformatics analysis of this sequence data to determine taxonomy and abundance. At all stages of this process there is bias introduced that can influence the results e.g. different DNA extraction protocols and 16S PCR primer sets used can dramatically affect the observed microbial diversity. Even the presence of relic DNA (DNA from dead cells in the sample) can lead to overestimation of microbial diversity and abundance (Carini *et al.* 2016). In our analysis we used the same DNA extraction and PCR amplification protocols across all samples. However, we cannot ignore the possibility that our dataset is likely to overestimate of the microbial diversity in the bulk soil and rhizosphere soil associated with *B. napus* due to the presence of relic DNA.

Our study demonstrated that no significant difference between the rhizosphere and bulk soil was observed under CT at flowering and harvesting stage, however minor separation was noticed at vegetative stage when illustrated through beta-diversity graphs. Chaparro *et al.* (2014) showed that rhizosphere bacterial communities at the seedling stage of *A. thaliana* were distinct from vegetative, bolting, and flowering stages but the communities associated with the later three stages were not significantly different than the bulk soil. Moreover, Bulgarelli *et al.* (2012) and Lundberg *et al.* (2012) also reported the higher assemblages between the bulk soil and rhizosphere in *A. thaliana*. In most plants species the release of vast quantities of photosynthates into the root zone is known to stimulate bacterial growth in, and attraction to, the rhizosphere. This leads to the so called ‘rhizosphere effect’, where the microbial population size and diversity is much greater in the rhizosphere than in the bulk soil (Bashir et al. 2016). The alpha diversity graphs showed that there was either no significant difference between the bulk soil and the rhizosphere or there was a significantly greater number of OTUs in the bulk soil. Although we have no data on population sizes, in terms of bacterial diversity we can conclude that WOSR has a negative effect on bacterial diversity in its rhizosphere. *Brassicas* do not form mycorrhizal associations, presumably by inhibiting the growth of

mycorrhizal fungi. *Brassicas* are known to exude glucoinolates many of which are known to have antimicrobial activity against Gram-positive and Gram-negative bacteria (Guil-Guerreroa *et al.* 2016). *B. napus* was found to produce progoitrin, glucoalyssin, gluconapin and glucobrassicapin (Velasco *et al.* 2008). The release of these compounds into the rhizosphere might explain the reduced bacterial biodiversity that we observed in the rhizosphere compared to the bulk soil.

Maintaining microbial diversity in the soil and rhizosphere is important in order to ensure functional redundancy to carry out essential ecosystem functions. However, we observed an overall decrease in bacterial diversity over the life time of the *B. napus* crop. A similar result was observed by Copeland *et al.* (2015) who found a decrease in bacterial diversity in canola. This is in contrast to other studies on cereals which found that there was a general increase in microbial diversity over the growing season (Gdanetz *et al.* 2017). We observed a statistically significant drop in the alpha diversity in the rhizosphere at the harvesting stage under ST compared with CT. These findings are further supported by our percent phylum distribution graph showing significantly higher relative abundance of bacterial phyla *Proteobacteria* and *Bacteroidetes,* and remarkably lower relative abundance of *Actinobacteria* and *Chloroflexi* only in rhizosphere at the harvesting stage compared to all other samples. Moreover, strip tillage practices persist distinct microbial communities in rhizosphere at plant maturity stage among the growth stages investigated, compared to conventionally tilled samples which was further supported by the beta diversity graph.

The beta diversity plots showed minor but clear separation between the CT and ST samples at vegetative stage which disappeared with plant development. Degrune *et al.* (2017) also found that tillage effect on the soil bacteria was stronger at early growth stage and was reduced at later growing stage. Interestingly, our percent family distribution graph demonstrated that bacterial diversity between the CT and ST was similar, however, the abundance of bacterial members between CT and ST was remarkably different in both bulk soil and rhizosphere at all growth stages. Dorr de Quadros *et al.* (2012) demonstrated that soil microbial diversity (in particular the anaerobes as *Verrucomicrobia, Firmicutes, Crenarchaeota, Chlamydiae, Euryarchaeota* and *Chlorobi)* through 16S amplicon metagenomic sequencing, and soil nutrients (phosphate, magnesium, total organic carbon and nitrogen) were significantly higher in no-tillage compared to conventionally tillage plots. Navarro-

Noya *et al.* (2013) observed similar results, in that under reduced tillage, the microbial biomass, plant organic matter and nutrient content were higher. These changes in the physical-chemical properties of the soil as a result of different tillage practices create different ecological niches that perhaps positively support the microbial proliferation in particular soil tillage. Yin *et al.* (2017) used the metagenomic approach to study the effect of conventional and No-till practices on the microbiome of wheat crops. They found that copiotrophic bacterial families such as *Oxalobacteriaceae, Pseudomonadaceae* and *Cytophagaceae* were more abundant in reduced tillage and oligotrophic bacterial families were more abundant in conventional tillage. However, Degrune *et al.* (2017) observed higher abundance of copiotrophic bacteria under conventional tillage and oligotrophic bacteria under reduced tillage. While tillage practices alter soil microbial community structure, the response of individual microbial groups appears to be very context-specific and cannot be generalized across various agroecosystems. The response is largely dependent on the soil physical-chemical conditions, soil types, climatic conditions and sampling depths.

Hale *et al.* (1971) reported that root exudation patterns can be affected by agriculture practices, soil water availability, stress, soil temperature, light intensity, degree of anaerobiosis, application of agro-chemicals, plant age, plant species, mineral nutrition, and soil microbes. Several studies demonstrated that root exudates and soil environmental changes can strongly affect the rhizosphere microbial community structure and composition over the life period of plants (Marschner *et al.*, 2002, Garbeva *et al.*, 2004, Cruz-Martínez *et al.*, 2012, Chaparro *et al.*, 2014, Van Horn *et al.*, 2014, Classen *et al.*, 2015). Our alpha diversity plots and family distribution graph showed that both rhizosphere and bulk soil microbiota were influenced by the developmental stage of the plant and that the 'rhizosphere effect' was evident in the root zone. Interestingly we observed that at harvesting stage in the rhizosphere, the number of bacterial families was higher than that in other growth stages under both tillage systems. Mendes *et al.* (2011) and Fang *et al.* (2016) reported that as a plant ages, it releases specific antimicrobial substances which can select for specific groups of resistant microbes. We also observed specific shifts in the microbial community at the harvesting stage particularly with bacterial families such as *Pseudomonadaceae Sphingobacteriaceae, Sphingomonadaceae, Flavobacteriaceae, Streptomycetaceae, Microbacteriaceae, Nocardiaceae*, and

Rhizobiaceae. Additionally, we observed that the composition of such bacterial members was remarkably higher in ST compared to that in CT. Gkarmiri *et al.* (2017) also reported similar bacterial families in rhizosphere of OSR through stable isotope high throughput sequence analysis.

Our results show that plant developmental stage and the rhizosphere effect lead to dynamic changes in the bacterial community structure in the root zone and that different tillage practices lead to lasting differences in bacterial communities both in the rhizosphere and in the bulk soil. We now need to try to understand, what consequences, if any, these changes in microbial community have on plant health/development and on important soil ecosystem functions.

Chapter 5

Structural variability and niche differentiation in the rhizosphere and endosphere bacterial microbiome of field-grown winter oilseed rape under soil tillage practices

5.1 **Introduction**

Land plants grow in soil, in direct proximity to a high abundance of microbial diversity (Tringe *et al.*, 2005). Plants and microbes have both adapted to use their close association for their mutual benefit. Therefore, plant-microbe interactions are of specific interest, not only to get a better understanding of their role during plant growth and development, but also for the effective manipulation of their relationship for applications such as phytoremediation, sustainable crop production and the production of secondary metabolites (Weyens *et al.*, 2009). The plant microbiome, often referred as the plant's second or extended genome, is one of the key determinants of plant health and productivity, by providing a plethora of functional capacities (Mendes *et al.*, 2011, Berg *et al.*, 2014). In plant-microbe associations, the bacterial microbiota in particular contribute strongly to plant nutrient acquisition, disease suppression, tolerance to (a)biotic stress, and influence the crop yield and quality (Weyens *et al.*, 2009, Lundberg *et al.*, 2012, van der Heijden *et al.*, 2015). In return, the host plant provides constant energy, carbon sources and niches for microbiota proliferation (Bulgarelli *et al.*, 2013).

Virtually all plant tissues host bacterial microbiota; at the soil-root interface (rhizosphere/rhizoplane), inside the plant tissues (endosphere), and at the air-plant interface (phyllosphere) (Beckers *et al.*, 2017). In recent years, high throughput sequencing approaches have provided detailed insights into the bacterial colonisation in different plant tissues for instance in *Arabidopsis* (Bulgarelli *et al.*, 2012, Lundberg *et al.*, 2012), *Populus* (Beckers *et al.*, 2017), maize (Peiffer *et al.*, 2013), rice (Edwards *et al.*, 2015), ryegrass (Chen *et al.*, 2016), wheat (Rascovan *et al.*, 2016), barley (Bulgarelli *et al.*, 2015), sugarcane (De Souza *et al.*, 2016). The microbiome inhabiting different plant compartments show different structures and functions. The rhizosphere soil at approximately 1-2 mm distance from roots is profoundly influenced by plant metabolism through the secretion of a great variety of exudates, including carbon rich photosynthates and antimicrobial compounds. This makes the rhizosphere a 'hotspot' environment, which accordingly results in a differentiation of the rhizosphere microbiome from bulk soil biota (Peiffer *et al.*, 2013, Schreiter *et al.*, 2014). The Rhizoplane is usually defined as a separate microhabitat from the rhizosphere, and is colonised by the microorganisms firmly attached to the root surface. The rhizoplane functions as a transitional boundary that

plays a critical gating role for controlling microbial entry into the plant root tissue (Edwards *et al.*, 2015, van der Heijden *et al.*, 2015). In contrast to the bulk soil and the rhizosphere, the endosphere features a highly specific microbiome, in which diversity is much lower than that estimated for microbiomes outside the roots (Bulgarelli *et al.*, 2012). Apart from the plant compartmentalisation effect on microbiome differentiation, other factors such as plant growth stages as well as field conditions, and agricultural management practices influence microbiota structure, function, diversity and composition (García-Orenes *et al.*, 2013, Degrune *et al.*, 2017).

Soil tillage is one of the anthropogenic activities that greatly alter the soil characteristics, including physical, chemical and biological properties (Jangid *et al.*, 2008). Therefore, different agricultural tillage systems influence the soil microbiota and their processes by changing the quality and quantity of plant residues entering the soil, their spatial distribution, soil pH, moisture, temperature, oxygen availability and also soil texture (Degrune *et al.*, 2017). Moreover, the growth and development stages of the crop are another major driver of microbial community structure in agricultural systems (Houlden *et al.*, 2008). As the root system develops over the growing season, there are corresponding changes in rhizodeposition which in turn change the dynamics of the microbial community (Philippot *et al.*, 2013).

A handful of research studies have explored the oilseed rape (*Brassica napus* L.) microbiome using high throughput sequencing techniques (De Campos *et al.*, 2013, Gkarmiri *et al.*, 2017, Rybakova *et al.*, 2017, Zhao *et al.*, 2017), despite the fact that OSR is the third largest source of vegetable oil, biodiesel (USDA-FAS, 2015) and the most profitable commodity crop (Teagasc-report, 2009). Additionally, OSR also an important break crop in cereal crop rotation that significantly reduce soil borne fungal infection and improve the yield of following cereal crop (typically up to 1.5t/ha approx.), which is higher than those achieved under continuous cereal operations (Teagasc-Report, 2017).

A deeper understanding of the structural composition of the bacterial microbiota present in different plant compartments, and especially the relationship between the below-ground and above-ground communities under the influence of soil tillage over the plant growth stages, may provide a deeper comprehension of the ecology and biology of WOSR bacterial microbiome. The hope is that this information can be utilised to develop new agronomic tools to promote eco-friendly

ways of WOSR cultivation with improved crop health and productivity. In the present work, the following two hypotheses were tested: (1) variations in the structure, composition and diversity of bacterial community occur across the WOSR compartments; rhizosphere soil, root and shoot at three different plant growth stages, (2) soil tillage is a major influencing factor contributing to the WOSR bacterial microbiome variations. To test these hypotheses, WOSR were grown under plough based conventional tillage and conservation strip tillage practices. The WOSR plant and bulk soil samples were sampled at three different WOSR growth stages; vegetative stage, flowering stage and harvesting stage. The structure, composition and diversity of bacterial community were investigated by means of deep MiSeq sequencing of the 16S rRNA gene amplicons.

5.2 Materials and Methods

5.2.1 Experimental site and design

The WOSR field experiment for microbiome study was conducted during the year August 2013 to July 2014 at Teagasc Crop Research Centre, Oak Park, Carlow, Ireland 52.86 ^{o}N and 6.92 ^{o}W. Samples for this study were taken from a field experiment evaluating the effect of crop establishment systems on the growth and development of WOSR. The establishment systems comprised of: 1) a conventional tillage (CT) plough based system and 2) a low-disturbance conservation strip tillage (ST) system. The WOSR variety 'Compass' was used. The detailed information regarding soil conditions and agronomic practices considered during this experiment are stated in Chapter 3, section 3.2.1 & Table A3.1.

5.2.2 Sample collection of bulk soil, rhizosphere and plant fractions

Bulk soil and plant samples were collected from the CT and ST treatments in triplicates from three blocks per treatment at three different plant developmental stages; vegetative stage (~120 days after sowing), flowering stage (~240 days after sowing), and at harvesting (~330 days after sowing) stage. Bulk soil samples were collected from a depth of 0-25cm, in triplicate from the edges of each block, using a hand auger. For each block, composite soil samples were prepared by thoroughly mixing the triplicate samples and a representative subsample of this was collected in sterile 50 mL Falcon tubes. The plant samples were processed into three plant microhabitat zones i.e. rhizosphere soil, root and shoot. The excess soil from the roots was removed by manual shaking, the soil attached to the roots was scraped off with sterile forceps into sterile 50 mL Falcon tubes. The root and shoot samples were washed separately in 50 mL Falcon tubes containing 30 mL of Phosphate Buffered Saline (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 7.0 pH, 0.02 % Silwet L-77) to remove the tightly adhered microbes from the surface followed by a sonication step (30 s at 50-60 Hz) as described by Lundberg *et al.* (2012). Samples were frozen using liquid nitrogen and stored in pre-labelled sterile 50 mL Falcon tubes. All the samples were stored in -80^{o}C until required for DNA extraction.

5.2.3 DNA extraction

For DNA extraction in soil, 0.25 g of soil was used for each individual DNA extraction. DNA was extracted using the MoBio PowerSoil^{TM} DNA isolation kit (Carlsbad, CA, USA) following the manufacturer's protocol. Total soil DNA was eluted in 50 µL of sterile water (Sigma Aldrich). For the plant DNA extraction, 0.25 g of plant tissues were individually ground into a fine powder in liquid nitrogen. Transfer up to 0.25 g of the resultant powder to a microcentrifuge tube. DNA extraction was performed using the same MoBio PowerSoil^{TM} DNA isolation kit (Carlsbad, CA, USA) following the manufacturer's protocol. Total plant DNA was eluted in 50 µL of sterile water (Sigma Aldrich). Concentration and purity of DNA was determined by Qubit 4 fluorimeter and Nanodrop spectrophotometry (Thermo Scientific, Wilmington, DE, United States) respectively. Post quantification, all DNA samples were normalized to 10 ng μL^{-1}.

5.2.4 Library preparation of 16S rRNA gene amplicon

The amplicon library of bacterial DNA was generated using the PCR primers 799F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAACMGGATTAGATACCCKG-3') and 1193R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGTCATCCCCACCTTCC-3') with Illumina adapter overhang sequences (as specified by underline) which covered ~400 bp of the hypervariable regions V5-V7 of the prokaryotic 16S rRNA gene (Bulgarelli *et al.*, 2015). The PCR reaction contained 25 µL of 2 x KAPA HiFi Hotstart ReadyMix, 1 µL of forward primer (1 µM), 1 µL of reverse primer (1 µM), 2.5 µL of DNA (~10 ng.μL^{-1}) and 20.5 µL of nuclease free H_2O in a total volume of 50 µL) by following the touch down PCR protocol (Table 5.1). After 16S PCR, 50 µL of reaction mixture was loaded on a 1.5 % agarose gel to separate the ~400 bp 16S rRNA gene amplicon from the ~800 bp 18S rRNA gene amplicon typically generated by the PCR primers 799F and 1193R. The smaller PCR product (16S rRNA gene amplified product; ~400 bp) was excised from the agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions. The concentration of the amplicon DNA in each sample was determined using the Qubit spectrophotometry (Thermo Scientific, Wilmington, USA) and normalised to an

equimolar concentration. Amplicons were generated, purified, indexed and sequenced with some modifications according to the Illumina MiSeq 16S Metagenomics Sequence Library Preparation protocol (16S-Metagenomic-library-prap, 2013). An initial PCR reaction contained 25 µL of 2x KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), 1 µL of forward and reverse primers (1 µM), 2.5 µL DNA (~10 ng μL^{-1}) and 20.5 µL of nuclease free H_2O in a total volume of 50 µL. The PCR reaction was performed using a 96-well Thermocycler (MJ research, USA) and the program mentioned in Table 5.1. All amplicons were cleaned using Ampure DNA capture beads (Agencourt- Beckman Coulter; Inc.) following addition of Illumina sequencing adapters and dual-index barcodes to each amplicon with the Nextera-XT Index kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions by following the PCR programme mentioned in Table 5.2. The amplicon libraries were pooled in equimolar concentrations. The final library was paired-end sequenced at 2 x 300 bp using a MiSeq Reagent Kit v3 on the Illumina MiSeq platform. Sequencing was performed on the Next Generation Sequencing Platform at Teagasc Moorepark research centre, Fermoy, Cork, Ireland.

Table 5.1 Touch down PCR programme for 16S rRNA gene amplification

Steps	Temperature (ºC)	Time (s)	# of cycles
01	94	120	
02	94	30	
03	58	30	X 5 cycles
04	72	15	
05	94	30	
06	57	30	X 5 cycles
07	72	30	
08	94	30	
09	56	30	X 5 cycles
10	72	45	
11	94	30	
12	55	30	X 20 cycles
13	72	60	
14	72	600	
15	15	Pause	

Table 5.2 PCR programme for dual-index barcoding amplification

Steps	Temperature (ºC)	Time (s)	# of cycles
01	94	120	
02	94	30	
03	55	30	X 10 cycles
04	72	60	
05	72	600	
06	15	Pause	

5.2.5 Amplicon data analysis

16S rRNA gene forward and reverse sequences were analysed separately using Usearch v9.2.64 32 bit www.drive5.com (Edgar, 2013) and Qiime, v1.9.0 (Caporaso *et al.*, 2010), unless otherwise specified the default parameters were used. The function -fastq_filter was used in Userach to discard sequences containing more than 4 expected base read errors. Singletons were discarded for further analysis. The retained high-quality sequencing reads then clustered into OTUs at 97 % sequence identity using the Userach pipeline. The "Gold" reference database (http://drive5.com/uchime/gold.fa) was used to identify and remove chimeras. Taxonomic classification of OTU-representative sequences was performed in Qiime using RDP classifier (Wang *et al.*, 2007) trained against the Greengenes database

(DeSantis *et al.* (2006), release 13_5). Likewise, we used OTUs' representative sequences to generate a phylogenetic tree in Qiime. The generated OTU table, taxonomy information and phylogenetic tree were used to implement the ecological and statistical analyses.

5.2.6 Statistical analysis

Data analysis and visualization were performed using Phyloseq (McMurdie & Holmes, 2013) package from R operated through R Studio v0.99.893. All OTUs belonging to chloroplast and mitochondria were identified and removed from the data set prior the analysis. For alpha diversity analysis, observed OTUs, Chao1 and Shannon indexes, normal distribution of the data were checked with the Shapiro-Wilk test. Significant differences in the variance of parameters were evaluated, depending on the distribution of the estimated parameters with ANOVA test followed by *post-hoc* significant test. For alpha diversity analysis, sequencing reads were rarefied at an even sequencing depth 6,781 (in forward sequences) and 7,665 (in reverse sequence) reads/sample respectively. To compare community diversity between the samples (beta-diversity), Hierarchical clustering and Principal Coordinate Analysis (PCoA) based on Bray-Curtis and Weighted UniFrac distances were calculated by using counts per million transformed OTU abundances. Permutational multivariate analysis of variance using distance matrices was performed in R using the 'adonis' function to define the proportion of variance explained by the factors microhabitat and/or tillage. We used a Venn diagram to visualise enriched OTUs, unique and shared, between plant compartments, growth stages, and tillage practices.

5.3 **Results**

5.3.1 Quality metrics of sequencing analysis

The microbiota of WOSR grown under two cultivation practices CT and ST were analysed at three different plant developmental stages; vegetative, flowering and harvesting stage. 16S rRNA sequencing libraries of the bulk soil, rhizosphere soil, root and shoot were prepared and sequenced. The sequencing of the amplicon libraries generated in a total of 2,945,778 raw reads. While performing the Illumina forward (R1) and reverse (R2) reads paired end merging, 998,647 (~33.90 %) reads got merged out of 2.9 M reads. Moreover, there were only 22,779 sequence reads retained upon quality filtering. Perhaps, this would occur due to the quality of the sequence reads which deteriorated towards the end of the sequences (Figure A5.1), specifically for reverse reads, where the PHRED score drastically reduced in both cases. These poor quality end reads caused an 'erroneous' base calling, and interfered with the merging of the forward and reverse reads. This in turn led to the production of singletons, which is generally reported to occur during the sequencing run when the sequencer went down and produced the sequences with low quality PHRED score. Therefore, we considered downstream analysis focused on either forward or reverse reads without paired end merging.

The analysis generated 2,945,778 sequence reads of which 1,705,310 (~57.89 % total forward sequence reads) and 1,467,292 (~49.81 % total reverse sequence reads) were retained upon quality filtering from forward and reverse reads respectively. These sequencing reads were clustered into 3187 OTUs (from the forward reads) and 3021 OTUs (from the reverse reads) at >97 % sequence similarity to microbial OTUs. After removal of chimeric OTUs, 2445 OTUs from the forward reads and 2109 OTUs from the reverse reads were retained for the downstream analysis. The detailed information regarding the quality metrics of forward and reverse sequence data presented in Table 5.3. Kwon *et al.* (2013) reported that reverse reads tend to have lower quality scores than forward reads. Additionally we recovered higher numbers of OTUs from the forward reads (2445 OTUs) compared to reverse reads (2109 OTUs). As a result, we considered the forward reads for the downstream bio-statistical analysis for this chapter. The OTUs which were not captured in forward sequencing reads but present in reverse sequence data (512 OTUs), and OTUs that were not captured in reverse sequence reads but present in

forward sequence data (850 OTUs) with the taxonomy information are detailed in Excel sheet_WS1 &2. The reverse sequence reads graphs (phylum distribution, alpha diversity graphs and beta diversity graphs) and bio-statistics analysis are also provided in the Appendix 5, Figure A5.2, A5.3, & A5.4 for comparative purposes.

To gain a detailed insight into the WOSR microbiome, data from the root, shoot and rhizosphere samples, in addition to the bulk soil samples were included in this study. The PCR primer set used (799-F and 1193-R) for the 16S rRNA gene amplification was capable of discriminating between plant-derived (e.g. plastids) and microbial-derived 16S rRNA gene sequences so they are suitable for the analysis of the WOSR microbiome. Therefore, under optimised PCR conditions, no mitochondrial 16S rRNA sequences were co-amplified from any of the plant compartments. Minor fraction of chloroplast/plastidial 16S rRNA sequences co-amplified was discarded from the dataset (Table 5.3).

Table 5.3 (A) Quality metrics of forward sequence reads.

Total number of reads and read lengths				
Total number of raw reads before QC	2,945,778			
Total number of assigned reads after QC	1,705,310 (~57.89 %)			
Read length	250 bp			
Total number of assigned OTUs (97 % sequence similarity)	2445 OTUs (after removal of the chimeric OTUs)			
Assigned reads	**Bulk soil**	**Rhizosphere soil**	**Root**	**Shoot**
Average number of reads	23913 ± 10652	24484 ± 11168	27773 ± 16556	26970 ± 15239
Non-target reads (%)	0.25 ± 0.04	0.28 ± 0.10	0.32 ± 0.08	0.38 ± 1.12
Average number of assigned reads	22984 ± 10233	23524 ± 10692	27255 ± 16157	26298 ± 14841
Normalised reads per sample	6781	6781	6781	6781
Average number of assigned OTUs	468 ± 11	519 ± 117	280 ± 18	153 ± 53
Unclassified reads (%)	0.04	0.04	0.02	0.03
% of total useable reads	24	24	24.50	24.25

(B) Quality metrics of reverse sequence reads.

Total number of reads and read lengths				
Total number of raw reads before QC	2,945,778			
Total number of assigned reads after QC	1,467,292 (~49.81 %)			
Read length	250 bp			
Total number of assigned OTUs (97 % sequence similarity)	2109 OTUs (after removal of the chimeric OTUs)			
Assigned reads	**Bulk soil**	**Rhizosphere soil**	**Root**	**Shoot**
Average number of reads	11646 ± 6399	12740 ± 6595	17630 ± 11398	18008 ± 11567
Non-target reads (%)	0.24 ± 0.05	0.27 ± 0.12	0.36 ± 0.07	0.40 ± 1.12
Average number of assigned reads	11639 ± 6396	12735 ± 6592	17534 ± 11288	17787 ± 11429
Normalised reads per sample	7,665	7,665	7,665	7,665
Average number of assigned OTUs	454 ± 42	489 ± 113	203 ± 14	151 ± 71
Unclassified reads (%)	0.07	0.05	0.45	1.25
% of total useable reads	24.41	24.20	24	24

5.3.2 Bacterial alpha diversity

Alpha rarefaction curves were constructed for each individual sample showing the number of observed OTUs, defined at 97 % sequence similarity cut-off in Qiime, relative to the number of total identified bacterial rRNA sequences (Figure 5.1). The bulk soil and rhizosphere samples exhibited diverse bacterial communities, whereas endophytic bacterial communities were much less diverse than rhizospheric and bulk soil communities as expected. Moreover, the endophytic samples (root and shoot) presented a higher degree of variation in the shape of their rarefaction curves, whereas bulk soil and slighty lesser extent rhizosphere soil displayed uniform rarefaction curves. Additionally, to assess the influence of tillage practices and growth stages within the WOSR bacterial communities in bulk soil, rhizosphere, root and shoot compartments, the alpha diversity indices were determined using the observed OTUs, Chao1 and Shannon (Figure 5.2A, B & C respectively, Excel sheet_WS-3 & 4). The dataset was rarefied to 6,781 sequence reads per sample before calculating diversity indices. The bacterial alpha diversity showed a decreased trend along a spatial gradient from the bulk soil to the endosphere (Figure 5.2). The soil profile (bulk soil and rhizosphere) presented greater richness and diversity compared to the plant samples (root and shoot). All three indices exhibited statistically significant difference between the compartments (ANOVA; $P > 0.001$), between the growth stages (ANOVA; $P > 0.01$) also in compartments*growth stage interaction (ANOVA; $P > 0.001$). However, there was no significant variation observed between the bulk soil and rhizosphere ($P < 0.05$) (Excel sheet_WS-4). Harvesting stage showed remarkable variation across the compartments under both tillage regimes especially between root and shoot microhabitats, where the shoot compartment presented the lowest bacterial richness, diversity and evenness compared to other growth stages. On the other hand, the significant difference between the tillage practices was only identified in observed OTUs (ANOVA; $P > 0.05$) and Chao1 (ANOVA; $P > 0.01$) but not in Shannon index (ANOVA; $P < 0.05$). Additionally, no significant difference in microbial diversity was observed in interactions of tillage practices*compartments or tillage*compartments*growth stages (ANOVA; $P < 0.05$) in all indices. However, Shannon index showed significant alteration in interaction of tillage*growth stages (ANOVA; $P > 0.05$) while this difference was not exhibited in diversity and richness indices.

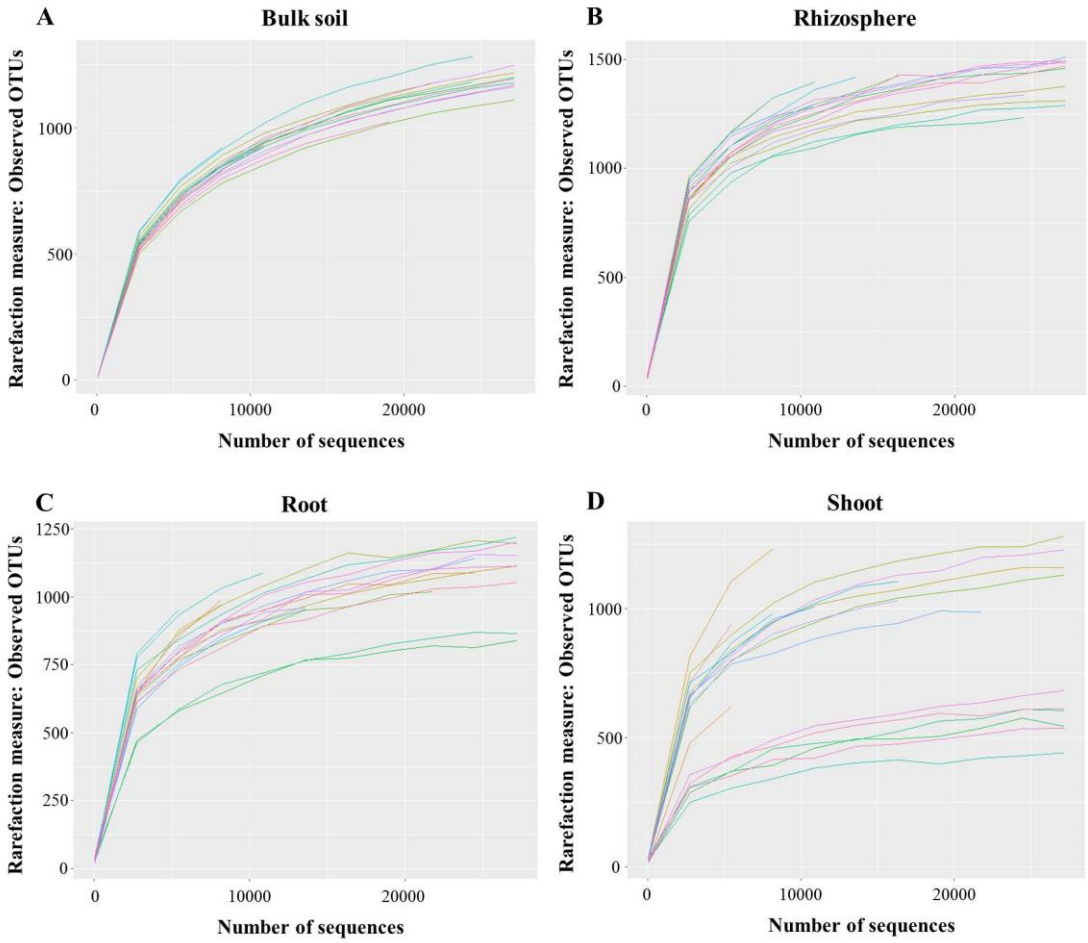
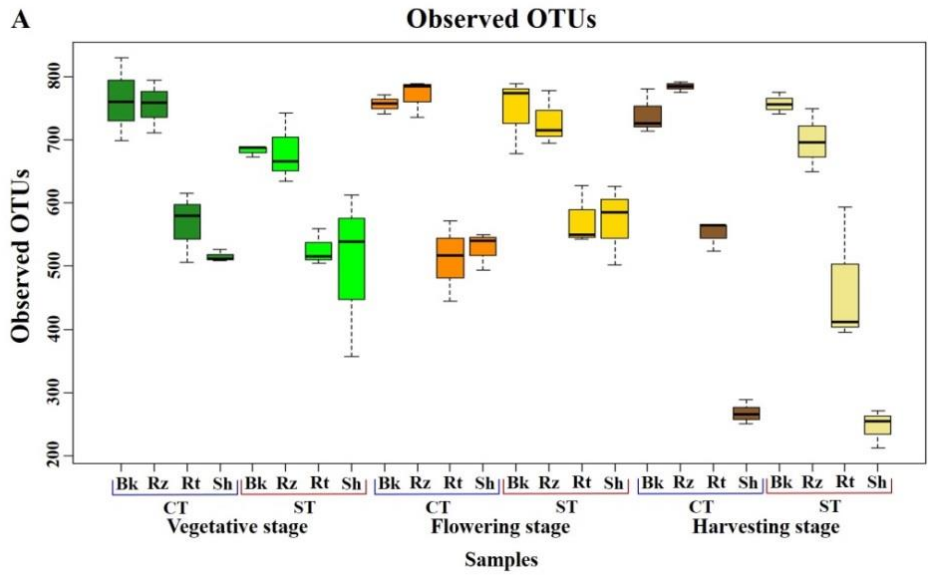
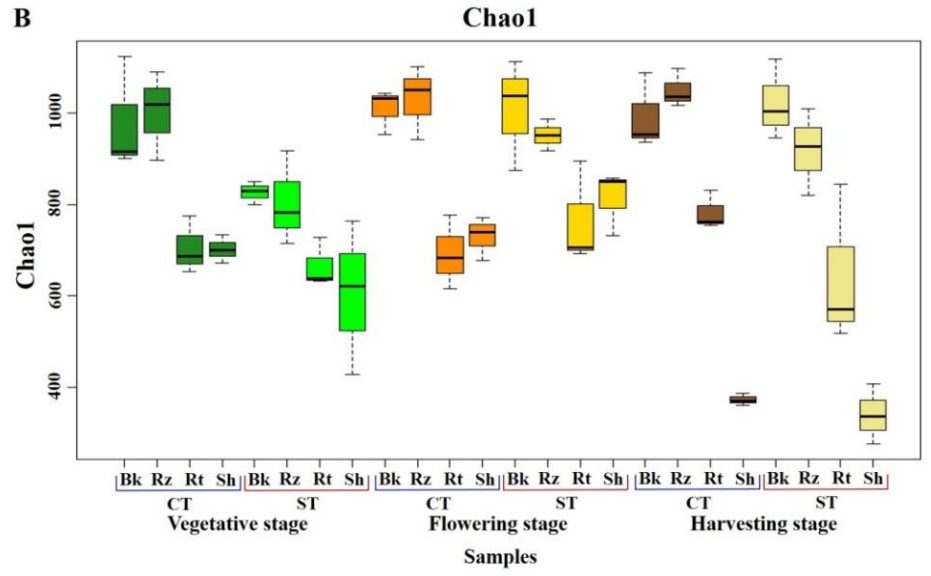
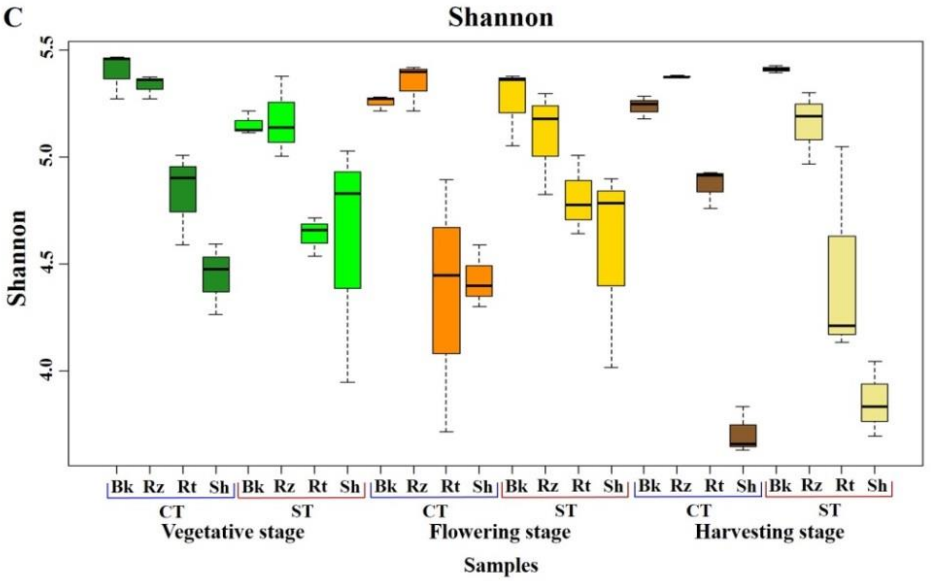


Figure 5.1 Rarefaction curves of the individual WOSR sample, grouped into different microhabitat zones; bulk soil (A), rhizosphere (B), root (C) and shoot (D), were calculated in Qiime based on 10,000 interactions. Rarefaction curves were assembled showing the number of OTUs, defined at the 97 % sequence similarity cut-off in Qiime, relative to the number of total sequences.


A
Observed OTUs
Observed OTUs
200
300
400
500
600
700
800
Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh
CT ST CT ST CT ST
Vegetative stage
Flowering stage
Harvesting stage
Samples


B
Chao1
Chao1
400
600
800
1000
Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh
CT ST CT ST CT ST
Vegetative stage
Flowering stage
Harvesting stage
Samples


C
Shannon
Shannon
4.0
4.5
5.0
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Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh
CT ST CT ST CT ST
Vegetative stage
Flowering stage
Harvesting stage
Samples

Figure 5.2 Alpha diversity analysis of the bacterial communities associated with bulk soil, rhizosphere root and shoot microhabitats under two tillage practices; CT and ST at three plant growth stages. The alpha diversity estimates; Total number of observed OTUs, Chao1 estimator and Shannon's diversity are displayed in A, B and C respectively. Sequencing reads of soil samples were rarefied at an even sequencing depth 6,781 reads/sample prior the analysis. Letters represent the bulk soil (Bk), rhizosphere (Rz), root (Rt), shoot (Sh), conventional tillage (CT), conservation strip tillage (ST). Statistical analysis of alpha diversity are displayed in supplementary excel file WS-3 & 4. Data are represented by three replicates of each sample type.

5.3.3 Bacterial beta diversity

To compare the composition of identified community members within different plant compartments and to find main factors driving WOSR bacterial community composition, beta diversity was calculated using Bray-Curtis and Weighted UniFrac dissimilarity matrices (Bray & Curtis, 1957, Lozupone *et al.*, 2011). Hierarchical clustering was also constructed based on both BC and WUF dissimilarities. These matrices were visualised using the Principal Coordinates Analysis (PCoA) as shown in Figure 5.3. The Hierarchical and PCoA analysis revealed strong clustering of bacterial communities according to the different plant compartments (rhizosphere soil, root and shoot) at both OTU and each phylogenetic level (Figure 5.3A-F). It revealed a clear separation between the root and shoot compartments and to a lesser extent between the bulk soil and rhizosphere microbiota. Partitioning of variance (ADONIS) based on the BC distance matrix at all growth stages indicated the significant contribution of niche differentiation ($P < 0.001$) and showed no influence of tillage practices ($P > 0.05$) with the exception of the harvesting stage ($P < 0.05$). Similarly, WUF analysis showed the significant contribution of microhabitat type to the clustering of WOSR microbiome at both OTU and phylogenetic level in all growth stages ($P < 0.001$; Figure A5.5). However, WUF failed to show soil tillage contribution at vegetative stage ($P > 0.05$; Figure A5.5A & B). While at flowering stage tillage practices showed minor impact ($P = 0.05$; Figure A5.5C & D) and at harvesting stage, tillage practices presented significant influence in microbiota niche differentiation ($P < 0.01$; Figure A5.5E & F). Furthermore vegetative (Figure 5.3A & B) and flowering stage (Figure 5.3C & D) presented no significant difference in interaction of tillage*compartments ($P < 0.05$) whereas harvesting stage (Figure 5.3E & F) revealed a significant influence of tillage*compartment interaction ($P < 0.01$). ADONIS based on BC and WUF analysis presented that microhabitat type, tillage practices and plant growth stages have significant contribution to the niche differentiation of the rhizosphere, root and shoot microbiota (R^2 and *P*-values are listed in Table 5.4).

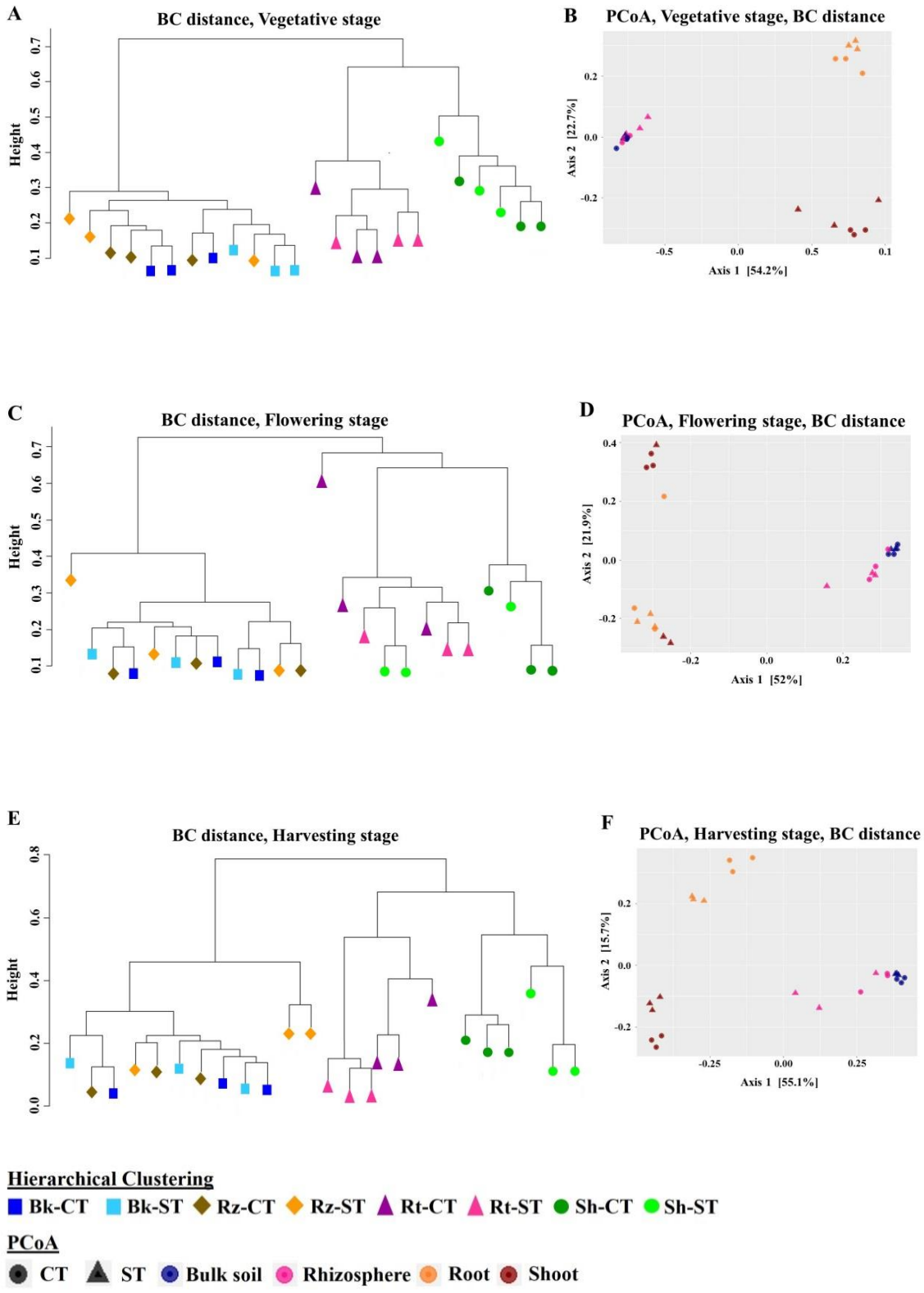

Figure 5.3 Beta diversity analysis of the bacterial communities associated with bulk soil, rhizosphere, root and shoot microhabitats under CT and ST tillage practices at different growth stages. Hierarchical clustering (A, C & E at vegetative, flowering and harvesting stage respectively) and PCoA (B, D & F at vegetative, flowering and

harvesting stage respectively) based on BC distance calculated using counts per million transformed OTU abundances. In hierarchical graphs shapes presented the different compartments and associated dark colours indicate the CT samples and light colour indicate the ST sample of respective compartment. Whereas, in PCoA graphs, colours define the compartments, while shapes depict the indicated tillage practices. Statistical results of beta diversity are displayed in Excel sheet_WS-5.

Table 5.4 Statistical analysis of beta diversity.

	Vegetative stage		**Flowering stage**		**Harvesting stage**	
	R^2	*P*	R^2	*P*	R^2	*P*
Bray-Curtis (ADONIS)						
Compartment	0.76314	**0.0002*** **	0.60987	**0.0002*** **	0.68774	**0.0002*** **
Tillage	0.02195	0.1348	0.03997	0.0719	0.04248	**0.0224***
Tillage*Compartment	0.02725	0.6315	0.06924	0.2279	0.09005	**0.0192***
Weighted UniFrac (ADONIS)						
Compartment	0.84080	**0.0002*** **	0.63188	**0.0002*** **	0.70056	**0.0002*** **
Tillage	0.02257	0.0649	0.04607	**0.0499***	0.08429	**0.002****
Tillage*Compartment	0.00891	0.9332	0.06517	0.2293	0.06849	**0.0359***

Significance levels: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

5.3.4 Structure of WOSR core microbiome

We further investigated whether the observed dissimilarity in bacterial community assemblages resulted from differences in WOSR microbial composition, abundance, or both. Therefore, to identify the factors driving WOSR core microbiome structure, the samples were grouped based on microhabitat zones (bulk soil, rhizosphere, root and shoot), plant associated compartments (rhizosphere, root and shoot), plant growth stages (vegetative, flowering and harvesting stages), and tillage practices (CT and ST). We first investigated the extent to which different microhabitat zones and plant associated compartments shared microbiota (Figure 5.4A & B respectively). The rhizosphere was the most similar to bulk soil, as indicated by the maximum shared OTUs (431 OTUs) were observed with bulk soil (Figure 5.4A). Moreover, there are noteworthy overlaps observed in OTUs between the plant associated compartments (Figure 5.4B). The OTUs identified in the rhizosphere were successful in colonizing the root, as rhizosphere and root compartments shared the higher number of OTUs (146 OTUs), followed by root and shoot shared the 79 OTUs, whereas shoot and rhizosphere shared only 33 OTUs. Furthermore, the result exhibited that rhizosphere showed highest richness in OTU count compared to their corresponding endophytic compartments. Likewise, the Venn diagram (Figure 5.4C) illustrated the complete outline of WOSR core microbiome over the different plant growth stages. The highest OTUs were shared between the flowering and harvesting stages (165 OTUs) followed by the flowering and vegetative stages (61 OTUs) and least for harvesting and vegetative stages (5 OTUs) were noticed (Figure 5.4C). This suggests that early growth stage WOSR core microbiome was dissimilar to the later growing stage. Furthermore, Figure 5.4D demonstrated the soil tillage impact on WOSR microbiome. Remarkably, the tillage practices shared 805 OTUs, however CT exhibited higher OTU richness (133 OTUs) compared to ST regime (57 OTUs).

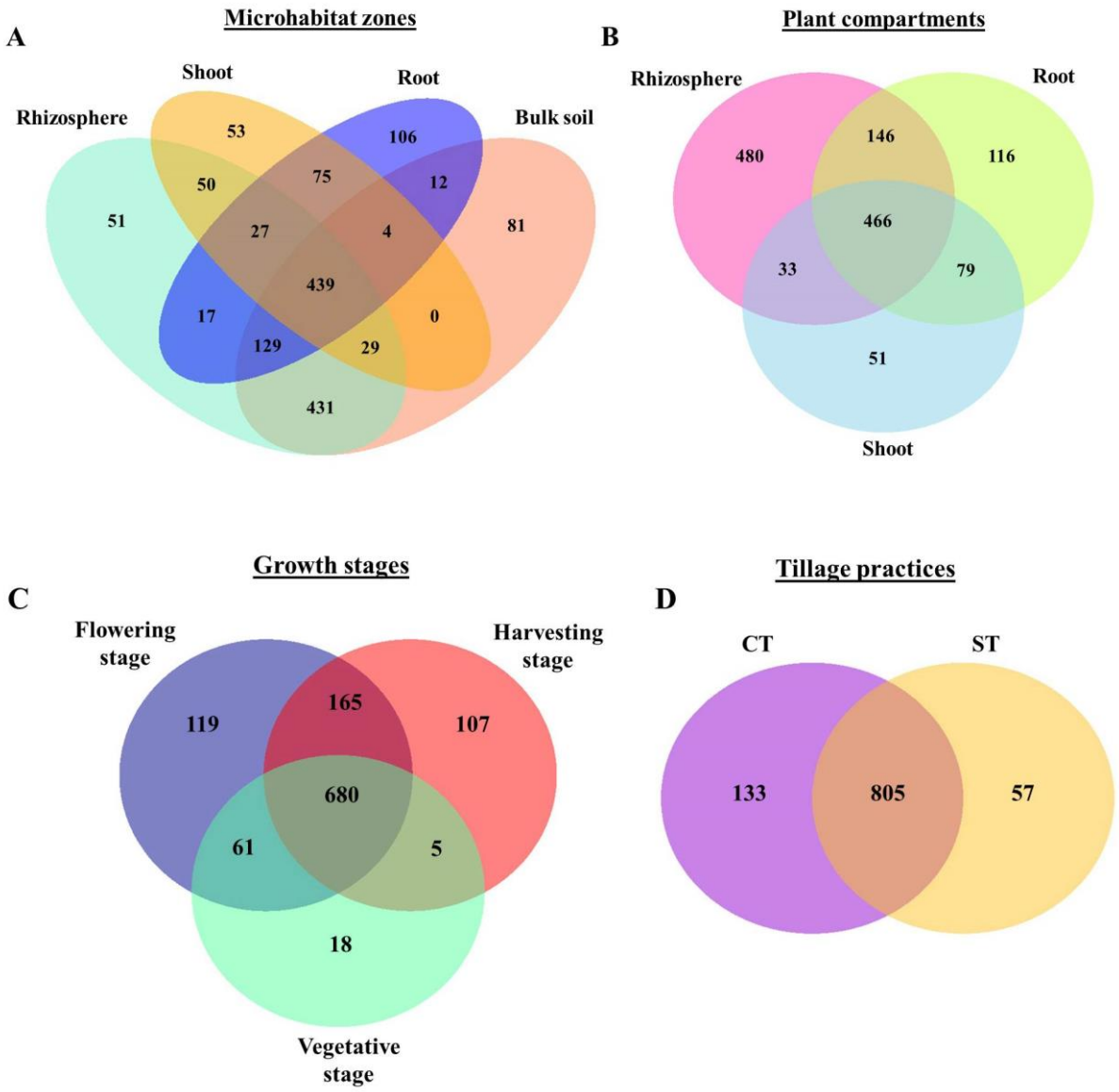

Figure 5.4 Venn diagrams showing number of shared OTUs between (A) microhabitat zones, (B) plant associated comartments, (C) growth stages and (D) tillage practices. Taxonomic information of shared and unique OTUs observed in each comparison were presented in Excel sheet_WS-6 to 9.

5.3.5 Taxonomic classification of WOSR core bacterial microbiome

Finally, we took a closer look at the individual bacterial phyla and OTUs, which differentiate the bacterial communities in microhabitat zones under the influence of soil tillage and plant growth stages. Taxonomic classification at the phylum level highlighted that Proteobacteria, Actinobacteria, Chloroflexi and Bacteroidetes largely dominate, and >80 % of sequence reads were assigned to these four phyla (Figure 5.5). In particular, the bulk soil and rhizosphere soil profile showed higher enrichment of phylum Actinobacteria whereas, root and shoot compartments presented higher abundance of bacterial members from phylum Proteobacteria. At the vegetative stage, there was no remarkable difference between the bulk soil and rhizosphere soil in terms of bacterial abundance or biodiversity. Nor were there obvious differences as a result of the two tillage practices. Likewise, noticeable difference in root under CT and ST was also not identified. The only exception to this was in the shoot under ST, which showed higher abundance of Bacteroidetes (4.33 %) compared to shoot under CT (1.2 %). At the flowering stage, the enrichment of phylum Bacteroidetes differentiated the rhizosphere from the bulk soil under both tillage practices. Moreover, the phylum TM7 was more abundant in plant microhabitats under CT (7.13 %) compared to ST (3.57%). Furthermore, Thermi differentiated shoot compartment of CT (7.01 %) to ST (1.81 %). At harvesting stage, the shoot microhabitat showed markedly distinct profile under CT and ST. The phylum TM7 (14.46 %) was highly abundant in shoot under CT whereas phylum Bacteroidetes (29.5 %) was remarkably enriched in shoot under ST. Moreover, the bacterial members from phylum Firmicutes noticeably distinguished the root compartment under CT and ST. Furthermore, at the phylum level, we evaluated all observed phyla with ANOVA to test the effects of bulk soil and plant compartments (bulk soil vs rhizosphere vs root vs shoot) on their percent relative abundance (Excel sheet_WS-10). There was no significant difference observed between the bulk soil and rhizosphere in any bacterial phyla ($P > 0.05$). The analysis showed that significant difference was mainly identified while comparing the soil profile to the endophytic profile. Root and shoot compartments were significantly differentiated to the bulk soil and rhizosphere virtually in all identified bacterial

phyla excluding Bacteroidetes. Phylum Chloroflexi significantly differentiate the root ($P < 0.001$), whereas shoot significantly differentiated by the phylum TM7 ($P < 0.001$) to the other remaining compartments.

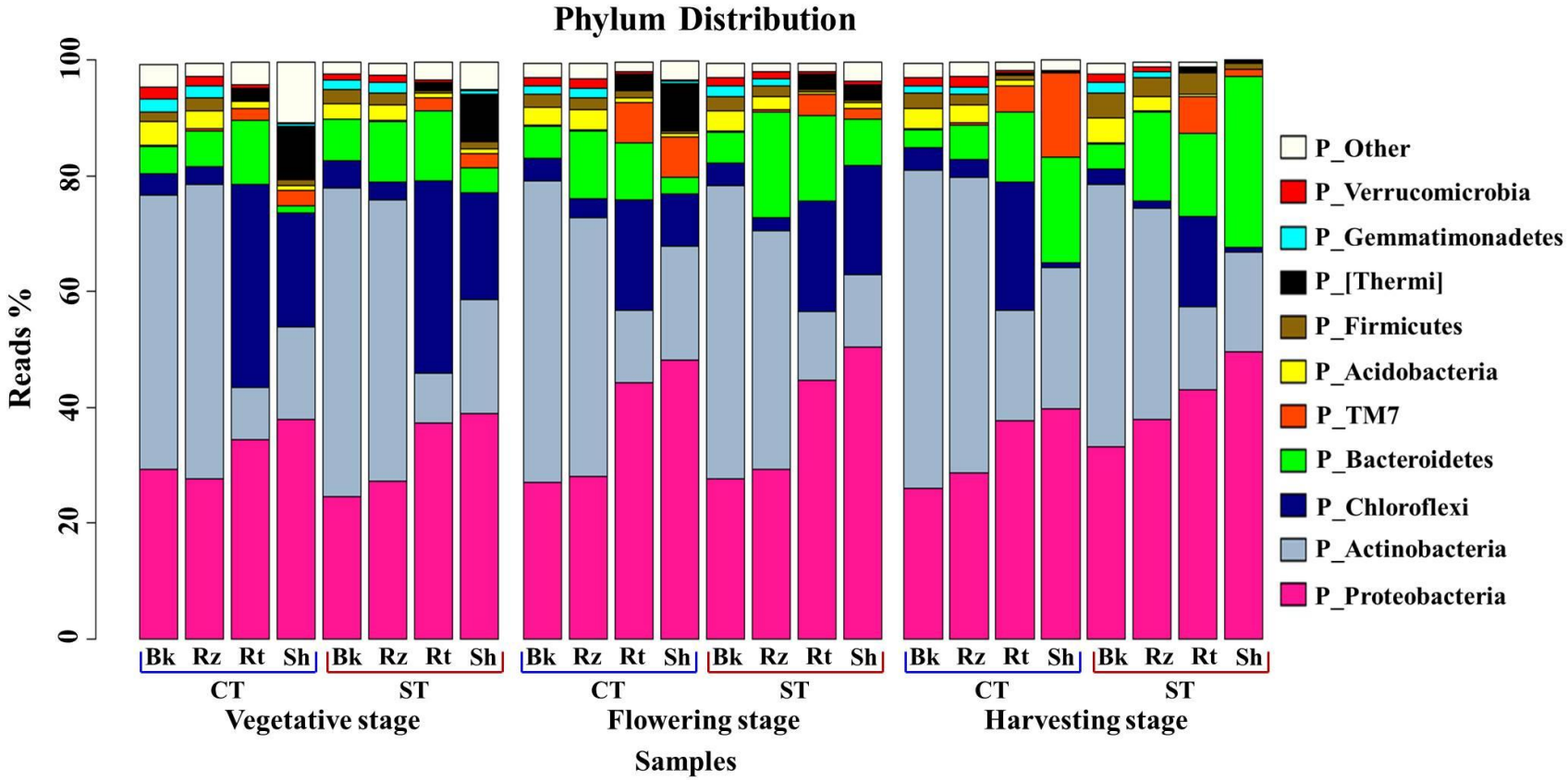

Figure 5.5 Percent relative abundance of bacterial phyla associated with bulk soil and WOSR rhizosphere, root and shoot compartments under conventional tillage (CT) and conservation strip tillage (ST) at three plant growth stages, are displayed in different colours. For each sample type, the number of replicates are n = 3. (Bk) bulk soil, (Rz) rhizosphere, (Rt) root, (Sh) shoot.

The core WOSR bacterial microbiome was presented by the 45 OTUs that were identified in all microhabitat zones throughout WOSR growth stages under both tillage practices (Excel sheet_WS11). The core OTUs represents the members of 24 bacterial families (exhibited in Excel sheet_WS12) that mainly compose the WOSR core microbiome. ANOVA was used to test the effect of microhabitats on the sequence counts of members of the core community. The Tukey's honest significant differences post hoc test was also performed. We observed significant microhabitat effects across all identified core bacterial families with the exception of family *Bacillaceae,* which was present in bulk soil and all plant associated compartments and showed the highest abundance compared to other families. In the rhizosphere soil, we observed a significant enrichment ($P < 0.05$) of *Comamonadaceae*, *Caulobacteraceae, Flavobacteriaceae, Xanthomonadaceae, Sphingomonadaceae*, and *Micrococcaceae* as compared to the bulk soil. Intriguingly, the bacterial family *Hyphomicrobiaceae* exhibited significant decrease trend in its abundance from bulk soil to shoot microhabitat. Moreover, significantly ($P < 0.05$) higher abundance of bacterial members from the families *Gaiellaceae, Mycobacteriaceae, Nocardioidaceae,* and *Micrococcaceae,* differentiate the soil profile (both bulk soil and rhizosphere) from the root and shoot microhabitats. On the other hand, significantly higher abundance of bacterial families such as *Pseudomonadaceae, Oxalobacteraceae, Methylophilaceae, Kouleothrixaceae, Comamonadaceae* and *Dolo-23* which distinguished root and shoot compartments from the soil profile (bulk soil and rhizosphere). The bacterial families *Kouleothrixaceae, Bradyrhizobiaceae,* and *Methylophilaceae* significantly separated the root compartment from the shoot while, *Microbacteriaceae, Nocardiaceae* and *Sphingomonadaceae* discriminated shoot from the root microhabitat ($P < 0.05$).

The bacterial families that represent >1 % of average relative abundance in the WOSR microbiome are shown in Figure 5.6. We observed that at vegetative stage the differences in bacterial relative abundance at family level were distinguishing the tillage practices. For example, ST system showed the higher abundance of bacterial families *Kouleothrixaceae* (14.72 %), *Comamonadaceae* (12.91 %), *Flavobacteriaceae* (9.62 %), *Gaiellaceae* (9.14 %), *Mycobacteriaceae* (8.77 %), *Sphingomonadaceae* (11.22 %), *Weeksellaceae* (7.28 %), whereas under CT *Nocardioidaceae* (7.76 %) and *Oxalobacteraceae* (11.04 %) were observed. At flowering stage, differences were observed between the two tillage practices in

rhizosphere, root and shoot compartments except for bulk soil. The enrichment of bacteria belonging to the families *Kouleothrixaceae* (10.77 %), *Flavobacteriaceae* (14.12 %), *Pseudomonadaceae* (12.52 %), *Sphingomonadaceae* (13.24 %) and *Comamonadaceae* (12.74 %) noticeably distinguished the microhabitats under ST compared to the CT. Moreover, at harvesting stage under ST, a higher abundance of bacterial members from the families *Enterobacteriaceae* (6.08 %), *Weeksellaceae* (13.58 %), *Pseudomonadaceae* (10.68 %), *Nocardioidaceae* (6.54 %), *Comamonadaceae* (8.1 %) and under CT *Gaiellaceae* (11.29 %) *Nocardiaceae* (11.81 %), *Oxalobacteraceae* (7.11 %), *Kouleothrixaceae* (12.9 %) were recorded which further differentiated the two soil tillage practices.

Some of the bacterial families were only present either in CT or in ST practices such as families *Methylophilaceae, Nocardioidaceae, Alteromonadaceae, Brucellaceae, Sanguibacteraceae, Xanthomonadaceae* were found in ST. *Aurantimonadaceae* was only observed under CT in shoot compartment. In root compartment families named *Bacillaceae, Micrococcaceae, Microbacteriaceae,* and *Sphingomonadaceae* were detected under ST while *Caulobacteraceae, Intrasporangiaceae, Solirubrobacteraceae* and *Sinobacteraceae* were present under CT practices. In rhizosphere, families Bacillaceae, Mycobacteriaceae, *Pseudomonadaceae, Sphingomonadaceae, Xanthomonadaceae,* and *Weeksellaceae* were only observed under ST differentiating it from CT system (Figure 5.6).

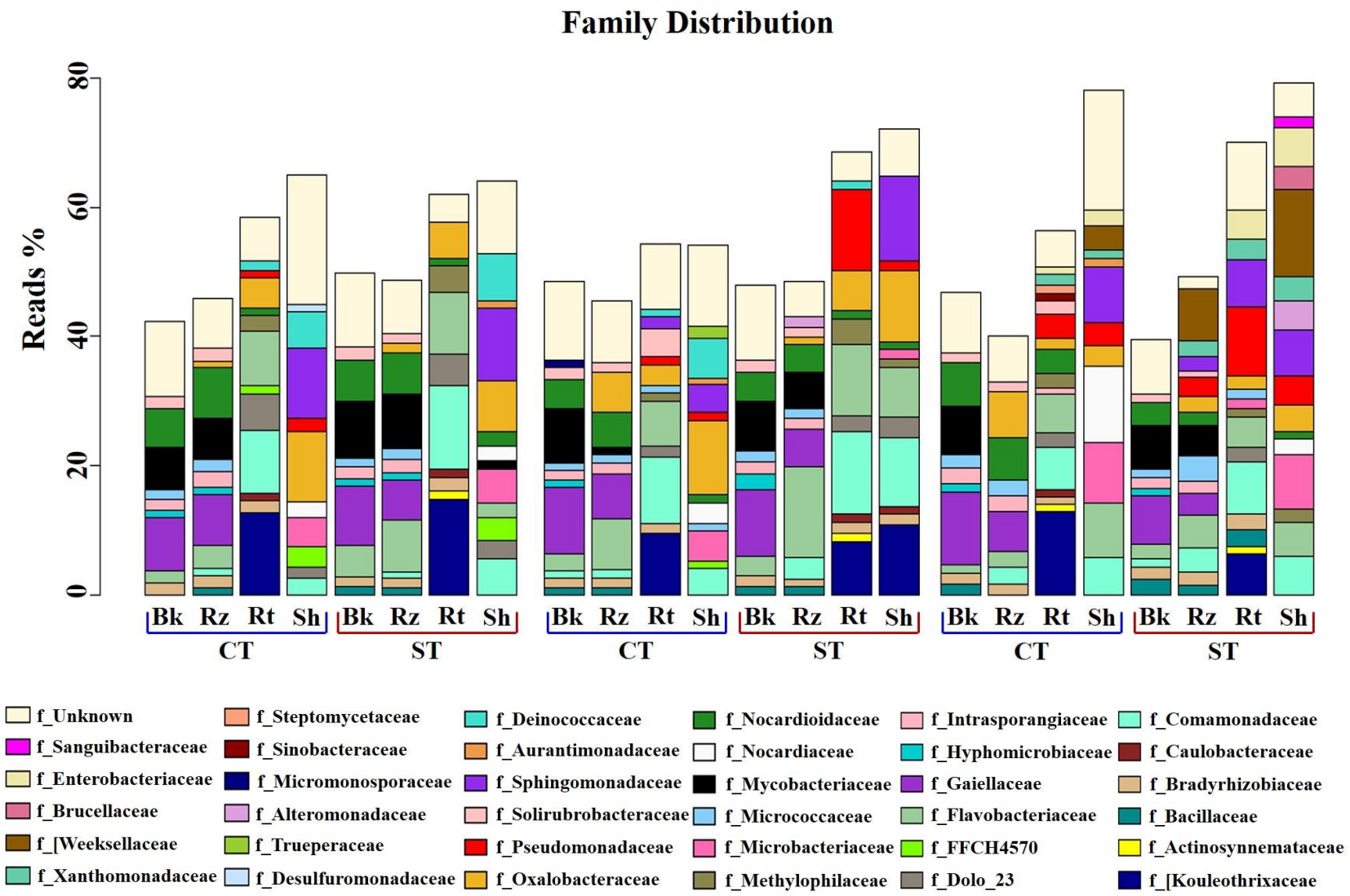
Family Distribution
Reads %
0
20
40
60
80
Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh
CT ST CT ST CT ST
f_Unknown
f_Sanguibacteraceae
f_Enterobacteriaceae
f_Brucellaceae
f_[Weeksellaceae
f_Xanthomonadaceae
f_Steptomycetaceae
f_Sinobacteraceae
f_Micromonosporaceae
f_Alteromonadaceae
f_Trueperaceae
f_Desulfuromonadaceae
f_Deinococcaceae
f_Aurantimonadaceae
f_Sphingomonadaceae
f_Solirubrobacteraceae
f_Pseudomonadaceae
f_Oxalobacteraceae
f_Nocardioidaceae
f_Nocardiaceae
f_Mycobacteriaceae
f_Micrococcaceae
f_Microbacteriaceae
f_Methylophilaceae
f_Intrasporangiaceae
f_Hyphomicrobiaceae
f_Gaiellaceae
f_Flavobacteriaceae
f_FFCH4570
f_Dolo_23
f_Comamonadaceae
f_Caulobacteraceae
f_Bradyrhizobiaceae
f_Bacillaceae
f_Actinosynnemataceae
f_[Kouleothrixaceae

Figure 5.6 Family-level relative abundance of bacterial communities across growth stage and crop management practices. The bacterial families that only represent >1 % average relative abundance of sequence reads in bulk soil and rhizosphere of WOSR under conventional tillage (CT) and conservation strip tillage (ST) practices at three plant growth stages; vegetative, flowering, and harvesting stages are displayed in different colours. The sequencing reads of samples were rarefied at an even sequencing depth 5,025 reads/sample prior to analysis. (Bk) bulk soil, (Rz) rhizosphere, (Rt) root, (Sh) shoot.

5.4 **Discussion**

Microbiome origin, dynamics and assemblage patterns are all important for the elucidation of its possible role in the plant growth, development and response to biotic, and abiotic stress (Turner *et al.*, 2013). There have been many previous studies on the characterisation of the root associated microbiomes (Bulgarelli *et al.*, 2012, Peiffer *et al.*, 2013, Bulgarelli *et al.*, 2015, Edwards *et al.*, 2015, Chen *et al.*, 2016). Bodenhausen *et al.* (2013) and Beckers *et al.* (2017) also characterised the leaf associated bacterial microbiota of *A.thaliana* and *Populus deltoides* respectively. Most of these studies have focused on the community characteristics of the general and specific microorganisms inhabiting plant associated compartments, their eco-system functioning and their responses to soil and plant factors (e.g. soil type, edaphic properties, plant species, cultivars). Here, we have characterised field grown WOSR microbiome (*viz*; rhizosphere, root and shoot compartments) at three plant growth stages, under the influence of soil tillage, using an Illumine MiSeq next generation sequencing survey of the amplicon 16S rRNA gene.

Initially, the OTUs rarefaction curves were estimated, which showed remarkable dissimilar shapes of the curves when comparing rhizosphre soil, root and shoot with bulk soil samples. Variation in the shapes of the rarefaction curves were the highest in shoot followed by root and at lesser extent to rhizoshere samples. High variability of OTUs richness in shoot and root compartments, as represented by the rarefaction curves, could possibly be driven by sporadic and non-unifrom colonisation of the bacteria in root and shoot microhabitats of WOSR. Our results are in agreement with the findings of Beckers *et al.* (2017) who found a similar trend of rarefcation curves in poplar bacterial microbiome. Furthermore, we found that alpha diversity estimates were highly dependent on the microhabitat zones, clearly differentiated the rhizosphere, root and shoot compartments by decreasing OTU richness and diversity. These results are in agreement with the general views of endophytic colonisation as reported by Beckers *et al.* (2017). The host plant deposits the root exudates in the root-zone which drive the chemotaxic attraction and bacterial colonisation in rhizosphere (Walker *et al.*, 2003, Reeder & Knight, 2010). After initial recruitment to the rhizosphere, only a subset of these microbes are bound to the root surface at the rhizoplane, suggesting selectivity for direct physical association with the root. This selection may occur by the plant, or may occur

through the ability to form biofilms (Edwards *et al.*, 2015). The rhizoplane serves as a critical gating role of the microbes which are attracted to the rhizosphere, while only a subset can bind the rhizosplane, and a fraction of these are permitted to enter and proliferate in the endosphere. Successful endophytic colonisation can require specific traits in bacteria such as expression of genes involved in chemotaxis, production of plant cell-wall degrading enzymes, formation of biofilm, flagella and pilli (Hardoim *et al.*, 2008, Bulgarelli *et al.*, 2012). After rhizoplane colonisation, adaptation to an endophytic lifestyle is dependent on the ability of the soil-borne bacteria to pass (actively or passively) the endodermis and pericycle, reach the xylem vessels and finally lead to systemic colonisation by certain bacterial species (Hardoim *et al.*, 2008, Compant *et al.*, 2010, Beckers *et al.*, 2017). The great loss of diversity and evenness from rhizosphere soil to endophytic compartments supports this view and indicates that only limited number of bacteria can adapt to an endophytic lifestyle and these bacterial members will therefore dominate endophytic assemblages (Beckers *et al.*, 2017).

This microbiome acquisition theory is further supported by the Venn diagram results, which presented the pattern of shared OTUs between the microhabitats (Figure 5.4B). Here decresing trend of unique OTUs were observed from rhizosphere to root followed by shoot compartment, whereas higher number of shared OTUs were observed between the rhizosphere-root followed by root-shoot and shoot-rhizosphere compartments. The result suggested that some of the bacterial members in WOSR shoot may come from the soil. Czajkowski *et al.* (2010) evidenced by using the GFP-tagged *Rhizobia* bacteria and reported that bacteria from the soil first colonise the roots and then migrate to the above ground part of the plant. Moreover, since, WOSR leaves are close to the ground during the vegetative stage, bacteria in the leaves may come from the rain splashing off the soil. A third explanation is that seed are colonised from the soil, and as the plant grows, bacteria colonise the expanding leaves.

To compare the bacterial community structures present in the plant compartments, we clustered all samples using hierarchical and principal co-ordinate analysis. At the phylum and OTU level, all samples strongly clustered according to the plant compartments and rendered microbiota significantly dissimilar from each other. Niche differentiation between rhizosphere and bulk soil biota failed to detect the rhizosphere effect in WOSR microbiome. These findings can also be supported

by the results from alpha diversity (Figure 5.1) where we did not observe a significant difference between the bulk soil and rhizosphere. Moreover, the Venn diagram result in Figure 5.4A also showed the maximum number of shared OTUs between the bulk soil and rhizophere. Similar results have also been observed in *A. thaliana* (belongs to the same family as OSR) where high assemblage patterns were identified between the rhizosphere and bulk soil over the plant life, in different soil types, also while testing the genotypes effect on microbiome (Bulgarelli *et al.*, 2012, Lundberg *et al.*, 2012, Chaparro *et al.*, 2014). However, root and shoot endophytic microbiota showed remarkable niche differentiation from the rhizosphere microbiota. Bulgarelli *et al.* (2013) proposed the two-step selection model for root microbiota differentiation from the rhizosphere where rhizodeposition and host-genotype dependent fine-tuning converge to select specific endophytic assemblages. Intriguingly, we also observed an interesting pattern of WOSR microbiota colonisation and niche differentiation over the plant growth stages. For instance, rhizosphere microbiome showed the minor but progressive trend of separation from the bulk soil microbiota. On the other hand, the WOSR endophytic microbiome (root and shoot) was distinct from the rhizosphere as well as from each other. However, few of the bacterial communities of root and shoot habitats moved across at flowering stage, and clear separation of root and shoot microbiota was observed based on both compartmental as well as soil tillage effects at harvesting stage. These results suggest that initially WOSR microbiota in each habitat colonised with taxonomically distinct microbial communities without any impact of soil tillage. However, from the flowering stage onwards, the endophytic microbiome started to develop its own specific profile under the influence of soil tillage which became significant at harvesting stage. These results are further supported by the Venn diagram of growth stages (Figure 5.3C) where higher number of shared OTUs were observed between flowering and harvesting stages. This suggests that soil tillage drives the WOSR microbiome composition, and growth stages fine-tune its structure.

Additionally, our hypothesis about the soil tillage has the significant influence on WOSR microbiome can further be supported by the result presented in the Venn diagram (Figure 5.4D). We observed that CT regimes comprised higher number of unique OTUs (133) compared to ST (57). Vian *et al.* (2009) reported that soil tillage practices are responsible for the alteration in soil edaphic factors such as temperature, aeration, moisture, structure, pH, soil nutrient. Moreover, under CT the

soil is inverted to a depth of about 25-30 cm, mixes the different horizons and break downs soil aggregates, which in turn provides good soil aeration that allows colonisation of minor or new species (Tilman, 1982). However under ST, soil is not inverted which leads to limited soil aeration and high soil moisture level that may create the anaerobic environment. Perhaps, this might be the reason of differential OTUs richness in CT and ST regimes.

WOSR core microbiome presented that between the four microhabitat zones (bulk soil, rhizosphere, root and shoot), 439 OTUs were shared. This suggest that soil was the main source of inoculum, and these bacteria were sucessful in colonising the WOSR rhizosphere, root and shoot microhabitats. At taxonomic level, WOSR core microbiome is mainly composed of the bacterial members from the phyla *Proteobacteria, Actinobacteria, Chloroflexi and Bacteroidetes* which further differentiates in to the families *Bacillaceae, Caulobacteraceae, Comamonadaceae, Kouleothrixaceae, Flavobacteriaceae, Gaiellaceae, Intrasporangiaceae, Methylobacteriaceae, Mycobacteriaceae, Micrococcaceae, Nocardiodaceae, Nocardiaceae, Pseudomonadaceae, Sphingomonadaceae, Weeksellaceae*, and *Enterobaceriaceae*. However, the composition and structure of this bacterial microbiome in plant associated compartments were varying in degree that largely controlled by the soil tillage practices and plant growth stages. For example, the Venn diagram in Figure 5.4C showed shared and unique OTUs pattern between the WOSR growth stages, where the lower number of shared OTUs observed with vegetative stage which indicate that WOSR bacterial microbiome at early growth stage was different than later growing stage. Moreover, some of the bacterial members were only observed at specific growth stage. Chaparro *et al.* (2013) reported that plant selects the subset of microbes at different growth stages by differing root exudation pattern during plant development. Furthermore, authors also observed that during the plant vegetative stage, sugars and sugar alcohol level was higher while, in later growing stage phenolics or amino acids were higher in root exudation, and this readily influence the microbial communities and modulate their transcription in different plant growth stages (Chaparro *et al.*, 2013).

Overall, our broader study of *B.napus* microbiome clearly confirms the microbial niche differentiation at the rhizosphere soil-root interface, along with a fine-tuning and adaptation of the endosphere microbiome in the shoot compartment. A unique ecological niche for the bacterial communities is also represented for each

plant compartment. Additionally, soil tillage and plant developmental stages further hone the WOSR microbiome structure and composition. As plant selects the subset of microbes at different growth stages for specific function, the soil tillage influences their availability in plant associated compartments. This study revealed a microbial community shift throughout the life span of the OSR plant which noticeably reflects the impact of agricultural practices such as tillage. Therefore, we suggest to carefully consider the agricultural practices that balance, contribute and maintain the enriched WOSR microbiome and overall metagenome function in-order to achieve a sustainable farming goal. Additionally, we believe that the assessment of microbiomes combined with network analysis may open new opportunities for targeted selection of biocontrol strains for a given host plant in a crop rotation regime. Such novel insights into the plant plus soil microbiome structure will enable the development of next generation strategies to combine both crop breeding and agronomic practices to address the current challenges in agriculture.

Chapter 6

Investigating the impact of soil tillage and oilseed rape-wheat crop rotation on the prevalence of 2,4-diacetylphloroglucinol producing *Pseudomonas spp.* in rhizosphere and root microhabitats using quantitative real time PCR

6.1 **Introduction**

The intensification of conventional agricultural practices is threatening ecosystem services and agroecosystem sustainability through soil erosion, agro-chemical pollution of groundwater, release of green-house gases and biodiversity loss (Tilman *et al.*, 2001). This is causing a paradigm shift towards sustainability, characterized by practices and concepts such as organic agriculture (Badgley *et al.*, 2007), agroecology (Rosset & Altieri, 1997), functional agrobiodiversity (Wood & Lenné, 1999), and conservation agriculture that comprises crop rotation and reduced tillage (Alvear *et al.*, 2005, Madari *et al.*, 2005, Rotenberg *et al.*, 2007). Conservation agriculture is largely motivated to improved crop yield, enhanced agro-ecosystem function in terms of increased soil fertility, maintenance of soil structure, augmented microbial activity, interruption of pest cycles, and weed suppression (Smith *et al.*, 2008). These processes are mediated largely by soil microorganisms through their complex biochemical processes (Kennedy & Smith, 1995).

Crop rotation may be the best, most widely practiced and cost-effective method for reducing the incidence of soil borne pathogen and improving crop productivity of subsequent crops (Larkin *et al.*, 2012). Conservation tillage minimizes the soil disturbance, and maintains the healthy and functional soil microbiota (Hobbs *et al.*, 2008). Moreover, crop rotation and conservation tillage can increase soil fertility and tilth, aggregate stability, improve soil water-nutrient management, reduce soil erosion and build-up of plant pathogens (Hobbs *et al.*, 2008).

Winter wheat and OSR are becoming a more increasingly popular crop rotation that provides a cash crop (OSR) in addition to the food crop (wheat), and reduces the incidence of take-all fungal disease (Angus *et al.*, 1991, Christen & Sieling, 1993) in the following wheat crop leading to an increase in wheat yield by ~20-30% (Kirkegaard *et al.*, 2008). Moreover, the deep penetrating tap root of OSR plants can improve soil structure and consequently aids in wheat root development (Kirkegaard *et al.*, 2008). A number of studies have demonstrated that wheat grown after OSR can extract more water and mineral nitrogen from the soil than wheat after wheat (Angus *et al.*, 1991, Kirkegaard *et al.*, 1994), and yielded 10-26% more than wheat monoculture or wheat in rotation with other cereals such as barley and oats (Christen & Sieling, 1993).

Wheat monoculture or rotation with other cereal crops can lead to an increase in the incidence of take-all fungus (*G. graminis*), leading to a decline of both crop yield and quality (Hornby, 1983). Moreover, other soil borne fungal pathogens such as *Pythium, Septoria, Rhizoctonia,* and *Fusarium* can also cause extensive damage to cereal plants, and they are major yield-limiting factors of cereal crops such as wheat and barley (Cook, 2001, Paulitz *et al.*, 2002). Root infection by these phyto-pathogens may be controlled by antagonistic effect of rhizosphere and root microbes. Numerous soil, rhizosphere and endophytic microbes have been shown to produce and release compounds that inhibit the growth of fungal pathogens. In particular strains of *Pseudomonas* spp. can protect plants from fungal diseases through a number of different mechanisms, mostly the production of antimicrobial compounds such as 2,4-DAPG, phenazine derivatives, pyrrolnitrin and pyoluteorin (Raaijmakers *et al.*, 1997). The antibiotic 2,4-DAPG has a wide-ranging biocontrol activity and is specifically active against the take-all pathogen (Kwak & Weller, 2013).

The polyketide antibiotic 2,4-DAPG has antibacterial, antiviral, antifungal, antihelminthic and phytotoxic properties as reviewed by Weller (2007). Moreover, 2,4-DAPG is reported to trigger induced systemic resistance (ISR) leading to enhanced plant protection (Iavicoli *et al.*, 2003). The genes involved in the biosynthesis of 2,4-DAPG are located on an 8-kb cluster. This region consists of eight genes *phlACBDEFGH*, and is conserved at the organizational level in 2,4-DAPG producing strains (Bangera & Thomashow, 1999, Delany *et al.*, 2000, Abbas *et al.*, 2002, Abbas *et al.*, 2004, Redondo-Nieto *et al.*, 2012). The key biosynthetic gene is *PhlD* (Bangera & Thomashow, 1999) which is required for the synthesis of phloroglucinol, a precursor of monoacetylphloroglucinol (MAPG) and 2,4 DAPG. *Pseudomonas* bacteria harbouring the *PhlD* gene for 2,4-DAPG production are found in soils worldwide (Raaijmakers & Weller, 1998, Weller, 2007). Moreover, *Pseudomonas* spp. are well-known as a plant growth promoting bacteria (Santoro *et al.*, 2015) therefore, their presence and abundance in rhizosphere and root can significantly improve plant health (Raaijmakers & Mazzola, 2012). The abundance and diversity of these bacteria may fluctuate according to soil management such as tillage and crop rotation (Rotenberg *et al.*, 2007), crop species and variety (Picard & Bosco, 2006), soil location (Meyer *et al.*, 2010) and soil geomorphology (Frapolli *et al.*, 2010). Additionally, the growing season of the crop is also a driver of bacterial population size in agricultural systems. Root system development over the growing season and associated changes in rhizodeposition may

alter the spatial distribution and quality of organic materials (Philippot *et al.*, 2013), influencing the dynamics of the microbial community over time.

Many research studies have reported the abundance of *Pseudomonas* bacteria that harbour the genes involved in the biosynthesis of antimicrobial compound such as 2,4-DAPG, phenazine, HCN and pyrrolnitrin in the bulk soil, rhizosphere and root interior (Raaijmakers & Weller, 1998, Weller *et al.*, 2002, Ownley *et al.*, 2003, Mavrodi *et al.*, 2012, Kwak & Weller, 2013, Pieterse *et al.*, 2014). These have shown to be effective for plant protection, as revealed for soil suppressive to take-all of wheat and barley caused by *G. graminis* (Weller, 2007), Fusarium wilt of pea mediated by *Fusarium oxysporum* f. sp. *pisi* (Landa *et al.*, 2002), *Thielaviopsis basicola* mediated black root rot of tobacco (Almario *et al.*, 2013), *G. tritici* and *Pythium ultimum* pathogen suppressive soil (Imperiali *et al.*, 2017). However, research investigating the combined impact of tillage practices and crop rotation on *PhlD*$^{+}$ *Pseudomonas* population density is limited. Therefore, the work detailed in this chapter has focused on understanding the contribution of tillage practices and crop rotation together on the prevalence of *PhlD*$^{+}$ *Pseudomonas* spp. in the rhizosphere and roots of WOSR and wheat (in rotation and in monoculture) crops at different plant growth stages, over two growing seasons, using quantitative real-time PCR (qPCR). We hypothesised that over the plant life cycle, WOSR - wheat crop rotation would show the substantial difference in 2,4-DAPG^{+} *Pseudomonas* spp. abundance in rhizosphere and root microhabitats under CT and ST systems.

6.2 **Materials and methods**

6.2.1 Experimental site

The winter OSR and wheat rotation field trials were conducted during the year 2014/15 and 2015/16 at Knockbeg, Carlow, Ireland (55.95 °N and 6.81 ºW).

6.2.2 Experimental design

The plant samples for this study were collected from a field experiment evaluating the impact of crop rotation along with crop establishment systems on the growth, development and production of cereal and break crops. The establishment systems comprised of (**1**) a conventional plough based system (CT), and (**2**) a low-disturbance conservation strip tillage (ST). These trials have been conducted continuously since 2012, where five different winter crops have been grown in rotation, for example OSR followed by wheat, oats, wheat, barley and again OSR under CT and ST tillage practices as described in Table A6.1. The growth period of each winter crop from sowing to harvesting was ~300 days.

The trials were a randomised block design where main-plots comprised the tillage practices and sub-plots divided into crop rotations. The individual plot dimension was 24 m x 4.8 m. The conventional establishment system comprised of mouldboard ploughing which inverted the soil to a depth of 230 mm, two days prior to sowing. The ploughed soil then received a secondary cultivation to 100 mm depth with a rotary power harrow. The OSR and wheat sown at 10 mm depth at row spacing of 125 mm using a conventional mechanical delivery, seed drill operated in combination with the power harrow. The low-disturbance establishment system deployed was a non-inversion system, comprised of a single cultivation/seeding pass of a rigid leg cultivator which were operated at 200 mm depth. These forward facing tines, with side ‘wings’ giving additional soil disturbance, worked directly in the cereal residue of the previous crop, disturbing approximately 50 % of the surface width between the legs. Seeding was by metered pneumatic delivery of seed to a point behind the cultivator leg, giving a row spacing of 600 mm in WOSR and 330 mm in wheat crops. Subsequent to seeding with both establishment systems, the soil surface was rolled using a ring roller. Crop management, other than crop establishment, followed standard practices for winter OSR

and wheat production in this region that illustrated in Table A6.2 and the basic soil analysis is presented in Table A6.3.

6.2.3 Sample collection and treatment

For this study, plants were sampled from three different rotational systems (1) WOSR (previous crop was barley), (2) wheat (previous crop was WOSR), and (3) from monoculture wheat (previous crop was wheat) under CT and ST tillage practices. Samples were collected in triplicate from each block of three replicated plots at three different growth stages; vegetative stage (~120 days after sowing), flowering stage (~240 days after sowing), and at harvesting (~330 days after sowing) stage in two sequential years; 2014-15 and 2015-16. Meteorological conditions during these years are presented in Table A6.4.

The plant samples were processed into two compartments i.e. rhizosphere soil and root. Roots were shaken to remove loose soil. Remaining attached soil (i.e. rhizosphere soil) was collected using sterile brushes in pre-labelled sterile 50 mL falcon tubes and stored at -80 °C until required for the DNA extraction. The root samples were washed twice with sterilized water followed by 30 mL of PBS buffer for 20 min at 180 rpm on a shaking platform. The roots were transferred to a new falcon tube and subjected to a second washing treatment (20 mins at 180 rpm in 10 ml PBS buffer). Doubled-washed roots were then transferred to a new falcon tube and sonicated for 10 min at 160 W in 10 intervals of 30 s pulse and 30 s pause (Bioruptor Next Gen UCD-300, diagenode, Liège, Belgium) to remove the tightly adhered microbes from the root surface. Roots were removed from PBS, rinsed in a fresh volume of 20 mL PBS buffer and grinded with mortar and pestle in liquid nitrogen. Pulverised roots were collected in 50 mL falcon tubes and stored at -80 °C until required for the DNA extraction.

6.2.4 Bacterial strain and culture storage

P. fluorescens F113 (Redondo-Nieto *et al.*, 2012) is a known 2,4-DAPG producer, was used as a source of the *PhlD* gene and as a positive control in the qPCR assays. The bacterial strain was cultured from -80 ºC frozen stocks. The strain was regenerated in King's B (KB) media. The plate was inverted and incubated for 24 hours at 28 ± 2 ºC. Single isolated colonies were removed from the plates and used to inoculate sterile KB

broth. The broth was incubated in rotary shaker for 24 hour at 120 rpm. To prepare the bacterial stock, the bacterial culture was transferred in sterile falcon tubes with sterilised glycerol having the final concentration of 40 % glycerol and 60 % 24 hour bacterial culture. This prepared mixture was transferred to a sterilised 1.5 mL eppendorf tubes aseptically and stored at -80 °C. Cultures were routinely re-cultured and prepared every 6 month.

6.2.5 DNA extraction and determining quality-quantity of DNA

Genomic DNA from *P. fluorescens* F113 was isolated from an overnight bacterial culture in KB medium. The Wizard® Genomic DNA extraction kit (Promega, USA) was used to extract DNA following the manufacturer's instructions and the extracted DNA samples were stored at -20 °C. DNA extraction from the rhizosphere soil samples were performed using the MoBio PowerSoil™ DNA isolation kit (Carlsbad, CA, USA) as per the manufacturer's guidelines. Total soil DNA was eluted in 50 µL of sterile nuclease free water (Sigma Aldrich). DNA extraction from plant root samples was performed from the ~0.5 g of plant tissues using 2 % CTAB method as per the protocol of Doyle (1990) and DNA was dissolved in 100 µL of sterile water. Concentration and purity of DNA was determined by Nanodrop spectrophotometry (Thermo Scientific, Wilmington, DE, United States). Post quantification, all DNA samples were normalized to 10 ng μL^{-1}. The three DNA samples from each microhabitat zone per block were pooled (e.g., the three DNA samples of rhizosphere soil samples from block 1 were pooled) to give representative DNA samples of rhizosphere and root from each block.

6.2.6 Generation of standard curve and *PhlD* quantification in samples

Quantitative PCR with SYBR green technology was used to quantify the *PhlD* gene copy numbers from the root and rhizosphere soil samples of WOSR and wheat plants. A standard curve of *PhlD* copy number was generated using genomic DNA of *PhlD*$^+$ *P. fluorescens* F113 bacterial strain. Genomic DNA of this bacterium was serially diluted ten-fold in three separated series to obtain standards from 3×10^6 to 30 fg DNA μL^{-1}. One microliter of each standard dilution (i.e. from approximately 4×10^5 to 4 *PhlD* copies) was used for qPCR analysis (Figure A6.1). QPCR assays were conducted using

96-well white microplates, Roche SYBR green master mix in a final volume of 10 µL, and a LightCycler 96 (Roche Applied Science, Meylan, France).

Table 6.1 Primers used for *PhlD* qPCR optimization

Primer	Sequence (5' – 3')	Amplicon length (bp)	Reference
B2BF	ACCCACCGCAGCATCGTTTATGAGC	319 bp	(Almario *et al.*, 2013)
B2BR3	AGCAGAGCGACGAGAACTCCAGGGA		

The reaction mixture contained 5 µL of Roche SYBR Green I Master Vial 1, 3 µL of Vial 2 (Roche Applied Science), 0.5 µL of primer B2BF (1 µM), 0.5 µL of primer B2BR3 (1 µM), and 1 µL of DNA. The final cycling programme included 10 min incubation at 95 ºC, 50 amplification cycles of 30 s at 94 ºC, 7 s at 67 ºC and 15 s at 72 ºC. Amplification specificity was checked by melting curve analysis of the amplification product using a fusion programme consisting of an initial denaturing step of 5 s at 95 ºC, an annealing step of 1 min at 65 ºC and a denaturing temperature ramp from 65 to 97 ºC with a rate of 0.11 $ºCs^{-1}$. Cycle threshold (Ct) of individual sample was calculated using the second derivative maximum method in the LightCycler 96 software v1.5 (Roche Applied Science).

The standard curve was obtained by plotting the mean Ct value of the three replicates (per DNA concentration) against the log-transformed DNA concentration. Amplification efficiency (E), calculated as $E = -1+10^{(-1/slope)}$, and the error of the method (Mean Squared Error of the standard curve) were determined using the LightCycler software v1.5 (Roche Applied Science). The equivalence between DNA amount and *PhlD* copy number was estimated based on (i) a *Pseudomonas* genome of approximately 7.26 fg DNA and (ii) the occurrence of one *PhlD* copy per genome. The detection limit was determined as the number of *PhlD* copies at the last DNA concentration giving 3 positive results out of 3 replicates. The amplification curve, melting curve and standard graph are displayed in Figure A6.1. Melting curve calculation and Tm determination were performed using the T_m Calling Analysis module of LightCycler 96 software v1.5 (Roche Applied Science). The standard curve thus generated from genomic DNA of *P. fluorescens* F113 was subsequently used as the external standard curve for determination of *PhlD* copy number in uncharacterised

DNA samples. The equimolar concentration (10 ng μL^{-1}) of DNA samples of root and rhizosphere soil were analysed by qPCR in triplicate (following the above protocol), and the mean Ct value was reported in the external standard curve to infer *PhlD* copy number in the sample, using the LightCycler 96 software and the 'standard curve' option for the absolute quantification. Positive control (*P. fluorescens* F113 genomic DNA 30 ng μL^{-1}), water control and three DNA standards from genomic DNA of *P. fluorescens* F113 (3000 pg, 30 pg and 0.3 pg corresponding to approximately 4 x 10^5, 4 x 10^3 and 40 *PhlD* copies) in triplicate were included as reference in each run to detect between-run variations.

6.2.7 Calculating copy number (CN) g^{-1} of soil and g^{-1} of root samples following qPCR

Sample concentration was determined following amplification cycle. The standard curve was developed in order to calculate the gene CN of 10 µL reaction of each dilution. The mean Ct value of each unknown sample was calculated using the equation line of the standard curve to get the estimated log value. The concentration was then back calculated to determine the CN g^{-1} of the original soil or root sample. The concentration of each DNA sample (both soil and root) was normalised to 10 ng μL^{-1}, and in each 10 µL reaction mixture 1 µL normalised DNA was used. Further information on these calculations are described here:

Example calculation for soil:

$$\text{CN } g^{-1} \text{ of soil} = \frac{(\text{Calculated CN X Original DNA concentration X dilution factor})}{\text{DNA concentration used for the reaction}} \text{ X } 4$$

- The calculated CN from the Ct value of the sample was 549
- Original DNA concentration of the sample was 86 ng μL^{-1}
- DNA was dissolved in 50 µL H_2O
- DNA concentration used for the each reaction was 10 ng μL^{-1}
- Soil used for the DNA extraction was 0.25 g

$$\text{CN } g^{-1} \text{ of soil} = \frac{(549 \text{ X } 86 \text{ X } 50)}{10} \text{ X } 4 \quad = 944{,}280$$

Example calculation for root:

$$\text{CN } g^{-1} \text{ of root} = \frac{(\text{Calculated CN X Original DNA concentration X dilution factor})}{\text{DNA concentration used for the reaction}} \text{ X } 2$$

- The calculated CN from the Ct value of the sample was 549
- Original DNA concentration of the sample was 108 ng μL^{-1}
- DNA was dissolved in 50 µL H_2O
- DNA concentration used for the each reaction was 10 ng μL^{-1}
- Root sample used for the DNA extraction was 0.50 g

$$\text{CN } g^{-1} \text{ of root} = \frac{(549 \text{ X } 108 \text{ X } 50)}{10} \text{ X } 2 = 592920$$

6.2.8 Statistical analysis

Statistical analyses were performed in R v3.4.2 operated through R Studio v0.99.893. The average values obtained from the three technical replicates of each qPCR assay were used for statistical analysis. Normal distribution of data was checked with the Shapiro-Wilk test. Significant differences in the variance of parameters were assessed, using non-parametric Mann-Whitney-Wilcoxon and Kruskal-Wallis tests to identify significant differences between the two tillage practices, years and between the crops. *Post hoc* comparisons were conducted by Kruskal-Wallis Dunn test. Statistical data is presented in Excel sheet WS-1 and -2. Data used for the heat map showing rankings of WOSR and wheat crops with their respective microhabitat zones of *PhlD*$^+$ *Pseudomonas* abundance at different growth stages under tillage practices, was normalised using the function 'scale' (R package 'stats') and graph was visualised using package 'heatmap.plus'.

6.3 **Results**

The abundance of bacteria harbouring the *PhlD* gene which is required for the biosynthesis of the antimicrobial compound 2,4-DAPG was quantified by qPCR in both rhizosphere soil and root samples of WOSR, and wheat crops grown in rotation and in monoculture under CT and ST practices. To our knowledge there is one copy of the *PhlD* gene per cell in the DAPG strains isolated to date. As such we make the assumption that one *Pseudomonas PhlD* gene represents one bacterial cell (Mavrodi *et al.*, 2007) however, this may not be the case in reality. In addition, this method counts dead and nonviable bacterial cells as well as the viable cells. Therefore our results may over estimate the number of DAPG producing cells in our samples.

6.3.1 Frequency of *PhlD*$^+$ in the rhizosphere of WOSR and wheat crops under different tillage systems

The *PhlD* gene copy numbers were quantified at three plant growth stages in WOSR, wheat in rotation, and monoculture wheat crops under CT and ST systems. The mean Ct value and associated calculated gene copy number per gram of each soil sample are presented in Table A6.5 & A6.6. At the vegetative stage, the copy number of *PhlD* gene in all studied crops was significantly higher (~1 to 1.5 log) in ST samples compared to CT samples (Figure 6.1A; $P < 0.001$). Furthermore, there was no significant difference in *PhlD*$^+$ microbial abundance between the crops as well as between the two years ($P > 0.05$). In both years the *PhlD*$^+$ *Pseudomonas* bacterial abundance was relatively stable in the rhizosphere of each crop under tillage treatments.

At the flowering stage in year-1, the *PhlD*$^+$ gene copy number in rhizosphere samples were similar in all crops ($P > 0.05$, Figure 6.1B). Whereas in year-2 samples, the *PhlD*$^+$ gene copy number significantly declined ($P < 0.05$) in WOSR by ~1 log, wheat (monoculture) by ~4 log under both tillage regimes, and in wheat (rotation) by ~3.5 log only in CT samples. In ST samples, *PhlD*$^+$ *Pseudomonas* abundance was relatively stable. Moreover, the *PhlD*$^+$ bacterial abundance was virtually identical in WOSR and wheat (in monoculture) crops in year-2 and wheat crop (in rotation) in year-1 under CT and ST tillage practices. There was a significant difference between the two years samples ($P < 0.001$) suggesting that *PhlD*$^+$ abundance at flowering stage was not stable in any crop.

While in the harvesting stage, *PhlD* gene abundance was relatively similar in both years ($P > 0.05$, Figure 6.1C). However, there was significant difference in *PhlD* gene copy number between the crops (year-1; $P < 0.05$ and year-2; $P < 0.005$) and, between the tillage practices ($P < 0.05$) in both the seasons. The abundance of *PhlD*$^+$ *Pseudomonas* spp. was markedly higher under ST samples compared to CT in each crop. Moreover, WOSR crop showed a significantly higher *PhlD*$^+$ bacterial abundance compared to wheat crops (in rotation and monoculture). Furthermore, the remarkable result was observed in *PhlD*$^+$ microbial abundance while comparing wheat grown in crop rotation and monoculture wheat under CT and ST. At harvesting stage, in rotational wheat crop, *PhlD*$^+$ bacterial presence was not identified in both years under CT, however under ST tillage regime *PhlD*$^+$ bacterial abundance was ~8 log per gram soil. Additionally, the wheat crop under monoculture exhibited the *PhlD*$^+$ bacterial abundance under both tillage regimes.

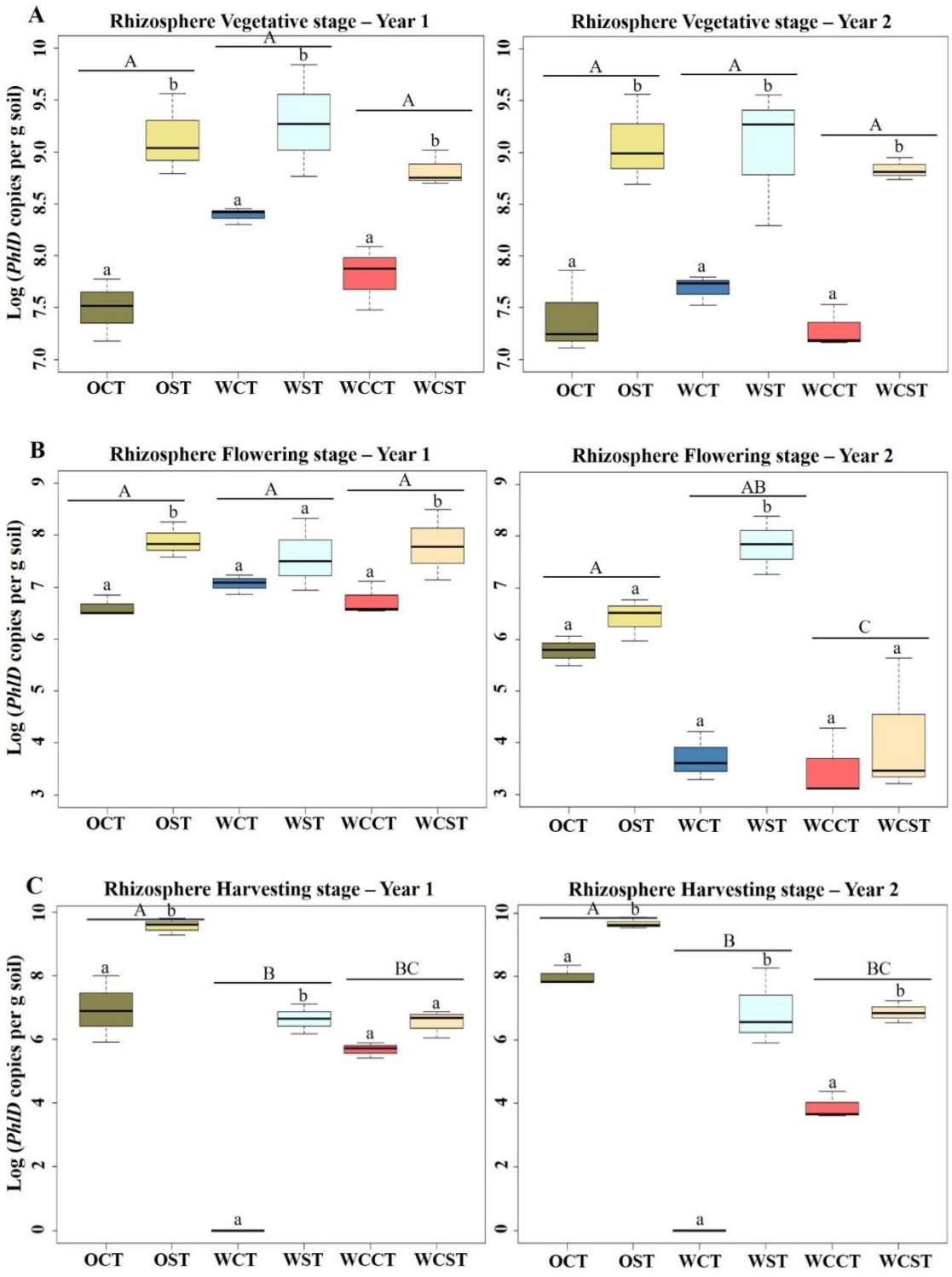

Figure 6.1 Population density of DAPG producing *Pseudomonas* in rhizosphere of WOSR and wheat crops at different plant growth stages. qPCR of *PhlD*$^{+}$ bacterial populations in WOSR and wheat rhizosphere, based on the number of *PhlD* gene copies detected per gram of rhizosphere soil sample at three plant growth stages (**A**) vegetative stage (**B**) flowering stage (**C**) harvesting stage under CT and ST in continuous two years

of crop rotation. *Uppercase* letters denote statistically significant differences by Tukey *post hoc* tests, $P < 0.05$, between the crops. *Lowercase* letters denote statistically significant differences by Tukey *post hoc* tests, $P < 0.05$, between the tillage practices within one crop. In box plots, darker colour defines the samples from CT and lighter colour defines the samples from ST. The individual symbols indicate the WOSR (O), wheat in rotation (W), wheat continuous (WC), conventional tillage (CT), conservation strip tillage (ST), samples collected in year 2014-15 (Year-1) and in year 2015-16 (Year-2).

6.3.2 Population density of indigenous *PhlD*$^+$ microbes in the roots of WOSR and wheat crops under different tillage systems

The *PhlD* gene copy numbers were tested at three plant growth stages in WOSR, wheat in rotation, and monoculture wheat crops under CT and ST systems. The mean Ct value and associated calculated gene copy number per gram of each root samples are presented in Table A6.7 & A6.8. At vegetative stage, the abundance of *PhlD*$^+$ microbes were significantly ($P < 0.05$) higher in the roots of WOSR under both tillage regimes especially in the ST samples compared to both wheat crops in year-1 (Figure 6.2A). Whereas in year-2, WOSR-ST samples showed a reduction of ~0.5 log *PhlD*$^+$ gene copy numbers compared to year-1 WOSR–ST samples. However, there was no significant difference between the samples of two years ($P > 0.05$). Both wheat crops (rotation and monoculture) under both tillage practices, and WOSR under ST showed virtually similar level of *PhlD*$^+$ population in both the years.

At the flowering stage, there was significant difference observed between the year-1 and -2 samples ($P < 0.05$, Figure 6.2B). The *PhlD*$^+$ bacterial abundance ranged between ~6.3 to 7.8 log in year-1, whereas in year-2 samples, the population was between log ~7.5 to 9.0. This suggests that in year-2 *PhlD*$^+$ bacterial population increased ~1 to 1.5 log in each crop. Moreover, the wheat crop in rotation exhibited no remarkable difference between the CT and ST samples in both years. Whereas, WOSR and wheat-rotation showed a higher bacterial abundance under ST samples compared to CT.

Finally, at harvesting stage, *PhlD*$^+$ bacterial population markedly increased in WOSR ~2.5 to 3 log under ST in comparison to CT samples ($P < 0.005$), and showed the highest abundance compared to other crops under both tillage practices (Figure 6.2C). Moreover, wheat-monoculture had a higher *PhlD*$^+$ populations under ST in comparison to CT in year-1. In year-2 the abundance of the *PhlD*$^+$ microbes increased under CT and reached a level similar to the ST samples at ~8.5 log *PhlD*$^+$ per gram of root. The wheat crop in rotation showed similar bacterial abundance under both tillage regimes in both years.

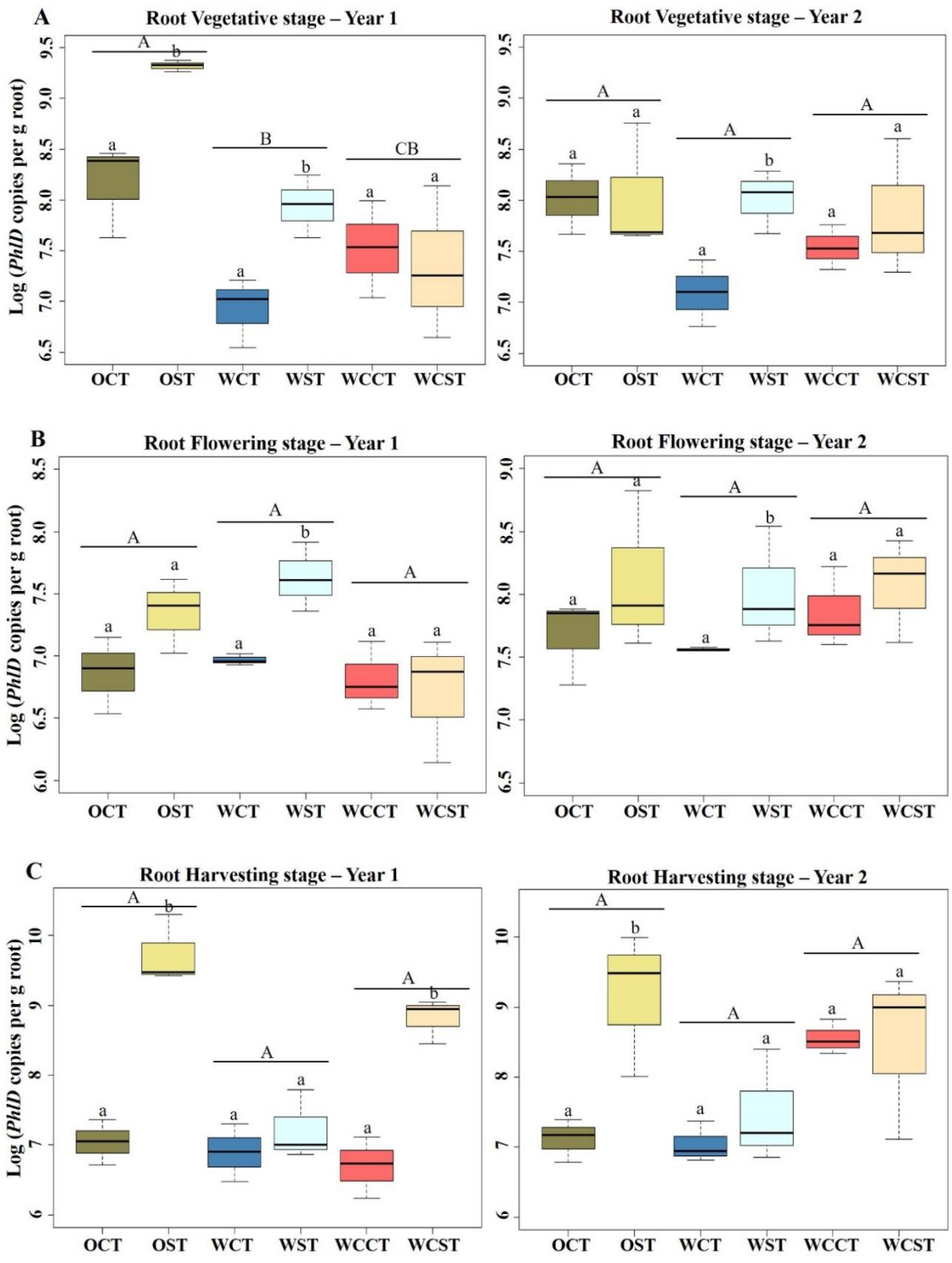

Figure 6.2 Population density of DAPG producing *Pseudomonas* in root of WOSR and wheat crops at different plant growth stages. qPCR of *PhlD*$^{+}$ bacterial populations in WOSR and wheat root, based on the number of *PhlD* gene copies detected per gram of root sample at three plant growth stages (**A**) vegetative stage (**B**) flowering stage (**C**) harvesting stage under CT and ST in continuous two years of crop rotation. *Uppercase*

letters denote statistically significant differences by Tukey *post hoc* tests, $P < 0.05$, between the crops. *Lowercase* letters denote statistically significant differences by Tukey *post hoc* tests, $P < 0.05$, between the tillage practices within one crop. In box plots, darker colour defines the samples from CT and lighter colour defines the sample from ST. The individual symbols indicate OSR (O), wheat after OSR (W), wheat continuous (WC), conventional tillage (CT), conservation strip tillage (ST), samples collected in year 2014-15 (Year-1) and in year 2015-16 (Year-2).

6.3.3 Relationships between microhabitat zones and crops of antimicrobial gene *PhlD*$^+$ abundance at different plant growth stages in two continuous years

The results obtained from quantification of the *PhlD* gene in the rhizosphere and root of WOSR, rotation wheat and monoculture wheat crops are displayed in a gradient map, based on the growth stages with sub groups of tillage and year (Figure 6.3). This graph shows three major clusters, where the first cluster consisted of both wheat crops (in rotation and monoculture) rhizosphere samples, the second cluster consisted of both wheat crops root samples and the third cluster comprised WOSR rhizosphere and root samples. The heatmap shows that wheat-rotation rhizosphere samples at vegetative stage, and WOSR root rhizosphere samples at vegetative and harvesting stages had the highest *PhlD*$^+$ bacterial abundance under ST. The vegetative stage showed the higher *PhlD*$^+$ bacterial abundance under both tillage practices in both microhabitat zones of wheat and WOSR crops compared to other growth stages. At flowering stage under the CT system, all the crops under both microhabitat zones showed similar *PhlD* gene copy numbers in year-1. However, year-2 in flowering stage exhibited a difference in *PhlD*$^+$ microbial population compared to year-1 in each crop's microhabitat zone except for root of wheat-rotation. Moreover at harvesting stage under the ST regime, all crops and their microhabitat zones showed highly stable *PhlD*$^+$ bacterial abundance in both years. Interestingly, the *PhlD*$^+$ bacterial population was the highest in WOSR rhizosphere/root followed by wheat-monoculture root, wheat-rotation root and then rhizosphere of both wheat crops under ST.

Furthermore, there was a clear trend identified in the rhizosphere of wheat-rotation under ST, where the *PhlD*$^+$ microbial populations were highest at vegetative stage which subsequently decreased at flowering stage followed by harvesting stage. Likewise, a similar trend was observed in the CT regime with a maximum *PhlD*$^+$ microbial population at vegetative stage, reduced at flowering but totally disappeared at harvesting stage. Wheat-monoculture showed a similar trend as wheat-rhizosphere under the ST regime, however under CT, the rhizosphere of wheat-monoculture responded differently than wheat-rotation, though the crop species and variety was same. Likewise, roots of both wheat crops under both tillage and all growth stages displayed different *PhlD*$^+$ gene copy numbers. Overall based on the crop species, WOSR showed higher *PhlD*$^+$ microbial abundance compared to both wheat crops.

Morover, conservation strip tillage showed a highly stable *PhlD*$^{+}$ microbial population in both years compared to conventional tillage at all growth stages.

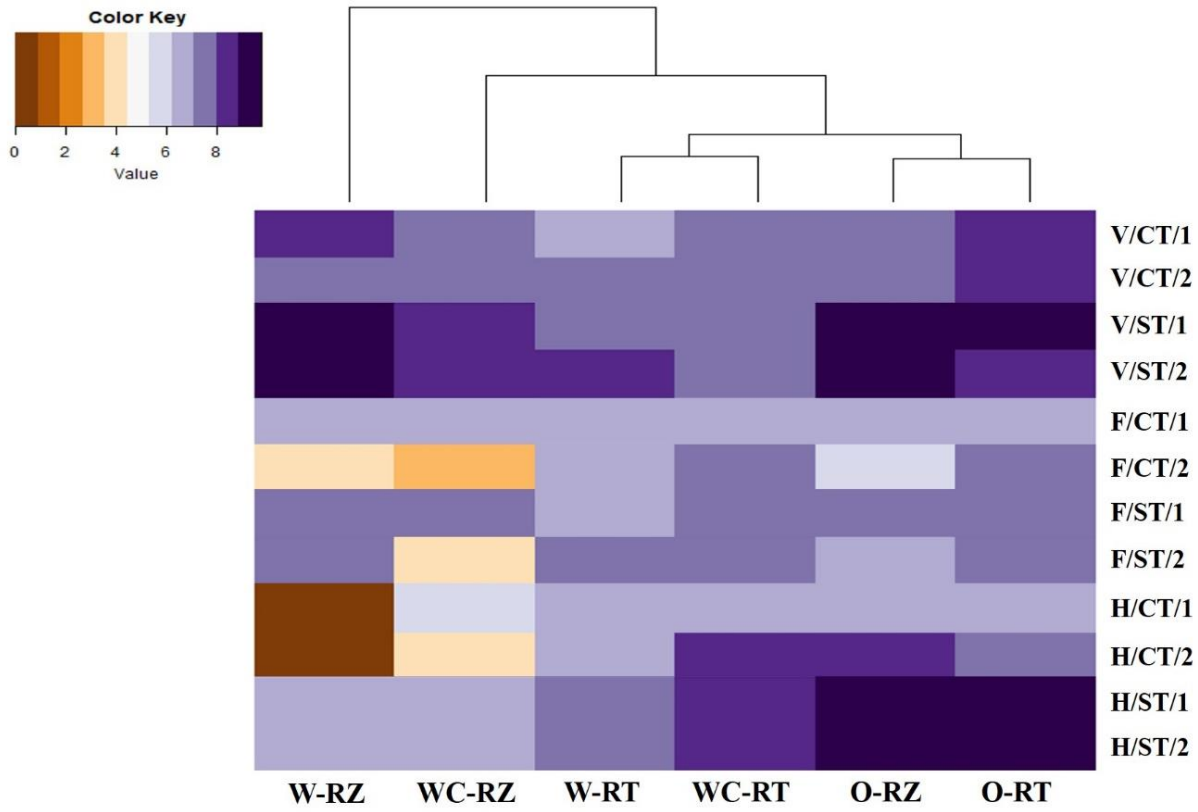

Figure 6.3: Heatmap showing normalised values of *PhlD* gene abundance in rhizosphere and root samples of WOSR and wheat plants under CT and ST at three different growth stages. The colour scale depicts lowest (brown) via intermediate (white) to highest (violet) values for each variable. The individual symbols indicate WOSR (O), wheat after WOSR (W), wheat continuous (WC), vegetative stage (V), flowering stage (F), harvesting stage (H), conventional tillage (CT), conservation strip tillage (ST), rhizosphere (RZ), root (RT), samples collected in year 2014-15 (Year-1) and in year 2015-16 (Year-2).

6.4 **Discussion**

Several studies have described the importance of microorganisms as suppressive agents against phytopathogens worldwide (Raaijmakers & Weller, 1998, Weller *et al.,* 2002, Almario *et al.,* 2013, Durán *et al.,* 2017, Imperiali *et al.,* 2017). However, the influence of different agricultural practices, in selection and proliferation of beneficial microbes such as *Pseudomonas* spp. in plant associated microhabitat zones remain under studied. Therefore, this current research was conducted to explore the combined impact of crop rotation (WOSR-wheat crop rotation and wheat monoculture), along with tillage practices (conventional tillage and conservation strip tillage), on DAPG producing bacterial spp. abundance in rhizosphere and root of WOSR and wheat crops at different plant growth stages over a period of two consecutive years.

Rotenberg *et al.* (2007) reported that farm management practices can influence overall structure of microbial communities in soil. Changes in soil populations might reasonably be expected to alter rhizosphere community structure to some degree and some of these rhizosphere microbes successfully colonise within the plant root zone through a multistep selection process, as proposed by Bulgarelli *et al.* (2015). Diverse soil and root-associated microbial populations affect crop health and productivity (Garbeva *et al.,* 2004). Here, in field experiments, the results showed that agriculture management practices significantly affected the incidence and relative abundance of *PhlD*$^+$ microbes in rhizosphere and root in subtle, complex but reproducible ways.

We observed that the DAPG producing bacterial abundance was overall higher under ST compared to CT. Moreover under ST, *PhlD*$^+$ microbial population was similar in both years in all crops especially at harvesting stage. These results are in agreement with the findings of Rotenberg *et al.* (2007). They represented a strong, positive correlation between *PhlD*$^+$ microbial abundance and conservation tillage compared to a conventional tillage system. Furthermore, Mavrodi *et al.* (2012) reported that the population size and plant colonisation frequencies of *PhlD*$^+$ *Pseudomonas* spp. was higher in wheat rhizosphere of irrigated field compared to dryland agriculture field. These findings may provide a possible explanation of our result that under conservation tillage regime perhaps soil moisture content was higher throughout the cultivation season compared to conventional tillage system, as

the soil was covered with crop residues, which reduces the soil evapotranspiration rate (Busari *et al.*, 2015). Therefore, this soil moisture level could be an influencing factor of *PhlD*$^+$ *Pseudomonas* spp. growth rate and competitiveness in soil tillage practices.

Degrune *et al.* (2017) stated that plant growth stages are the one of the major drivers of microbial community structure in agricultural systems. This was showed that the *PhlD*$^+$ population in rhizosphere and root were not consistent during all plant developmental stages in both WOSR and wheat crops. These changes may be associated with the growth of root and related variation in rhizodeposits over the plant developmental stages (Philippot *et al.*, 2013). Furthermore, we observed that *PhlD*$^+$ bacterial population dynamics did not change in both years at vegetative and harvesting stages however, relative alterations in *PhlD*$^+$ bacterial population was noticed at flowering stage while comparing both years. Probable explanation of these results might be that the microbial population could also be determined by the environmental conditions such as temperature, humidity and rainfall (Classen *et al.*, 2015) which influence the belowground plant-microbe and microbe-microbe interactions. At flowering stage, we observed that in climate and weather data, the environmental conditions were not consistent in both years and perhaps this could be the reason behind the *PhlD*$^+$ bacterial population dissimilarity. Furthermore, we found that *PhlD*$^+$ bacterial population dynamics over the growing season was also tillage dependent. Degrune *et al.* (2017) stated the possible reason of microbial alteration due to tillage is that the establishment of root system under CT and ST over the growth period might differ, which in turn may influence the water, nutrient flows and oxygen availability through the soil profile.

The heat-map analysis resulted in three different clusters comprising wheat rhizosphere, wheat root and WOSR root-rhizosphere. Picard & Bosco (2006) stated that alteration in population, diversity and colonisation pattern of *PhlD*$^+$ *Pseudomonas* spp. in rhizosphere and root habitats are determined by the crop species and varieties. The root associated microbial density and diversity has been related to differences in the root system architecture. The root structure of the wheat and WOSR crops are fibrous and tap root system respectively. Therefore, the difference in root morphology of wheat and WOSR may lead the two different clusters based on the crop species. On the other hand, the wheat crop formed two clusters based on the microhabitat zones. Bacterial colonisation frequency in

rhizosphere and in root interior is different. The rhizodeposition and root exudation by the host-plant in the root zone fuels chemo-attraction to colonise the rhizosphere from surrounding bulk soil. The rhizosphere soil-root interface acts as a selective barrier that limited the endophytic competence/colonisation efficiency of the microbes. Therefore, the niche differentiation of the rhizosphere and root microbes formed two different clusters in the resulted heat-map.

Nonetheless, we noted recurring patterns of responses to agricultural practices across the crops and plant growth stages. For example, we found no difference in overall *PhlD*$^+$ *Pseudomonas* spp. abundance in wheat grown in rotation and wheat monoculture. Kwak & Weller (2013) stated that cereal crops grown continuously in the same soil can develop disease suppressive potential for the soil borne diseases after a few years and indicated that this type of soil comprises a higher relative abundance of *PhlD*$^+$ *Pseudomonas* spp. This might be the reason that monoculture wheat has a higher abundance of *PhlD*$^+$ microbes compared to rotational wheat. Likewise, the wheat crop under rotation showed a substantial difference at harvesting stage between the CT and ST regimes. For example, the *PhlD*$^+$ bacterial population in the rhizosphere of the wheat crop was absent at harvesting stage under CT. On the other hand, under the ST regime, the bacterial population was more than 6 log higher in wheat rhizosphere. However in monoculture wheat, *PhlD*$^+$ bacterial population was present under both tillage practices at all plant growth stages. These results may indicate a negative impact of crop rotation on bacterial manifestation in wheat grown under CT. Though, to decide what was the main responsible factor for this result either of crop rotation or CT system was challenging.

In the present study, WOSR was used as a break crop in wheat crop rotation. The results indicated that WOSR comprised a *PhlD*$^+$ *Pseudomonas* population between 10^7 to 10^9 per gram of soil and root in rhizosphere and root at all growth stages, though the microbial abundance was higher in ST compared to CT. Sturz & Christie (1995) and Sturz *et al.* (1998) stated that after harvesting the crop, the roots remain in the soil until the next crop cultivation, and release the microbes which could lead the significant accumulation of bacteria in soil over time and that transferred from one crop to the next under crop rotation scheme. Moreover, plants make a selection of the microbes in their rhizosphere from the surrounding soil by producing the specific compounds in rhizodeposits at different growth stages which

is evidenced through many research studies (Chi *et al.*, 2005, Bais *et al.*, 2006, Bulgarelli *et al.*, 2012, Edwards *et al.*, 2015). Based on these theories, WOSR seem to be an effective break crop in wheat crop rotation, and the data also suggested that tillage practices were the driver of *PhlD*$^+$ microbial alteration. Vian *et al.* (2009) reported that tillage practices bring the physicochemical changes in soil that may change the microbial population in soil overtime. Therefore, we hypothesised that under the CT impact, multiple factors may be responsible for example, root exudates produce by the wheat crop under CT, other microbial interactions, nutrient pool, water availability, soil physiochemical and environmental conditions for the rapid drop of *PhlD*$^+$ *Pseudomonas* population in wheat rotation at harvesting stage.

The results of the present study suggested that to gain a profound understanding between the links of tillage practices, crop rotation, soil characteristics and abundance of *PhlD*$^+$ bacterial population, future studies should include a detailed soil physicochemical analysis, root exudates patterns over the plant life, use of other break crops in wheat rotation and record of disease incidence. These studies would help to show up the potential of *Pseudomonas* bacteria as bio-indicator of soil through crop management practices.

Chapter 7

General Discussion

The past decade has clearly been a golden age for microbiome research. Since the discovery of the associations between eukaryotic and prokaryotic cells, it has quickly become one of the most studied research areas. Numerous studies have been investigating eukaryotic-prokaryotic associations: from the human microbiome (The Human Microbiome Project, 2012) and host-genotype associations therein (Spor *et al.*, 2011, Koch, 2014), gut microfauna of insects (Sudakaran *et al.*, 2012, Hansen & Moran, 2014) to microbiota associated with plants (Lundberg *et al.*, 2012, Bulgarelli *et al.*, 2015, Edwards *et al.*, 2015, Beckers *et al.*, 2017). The microbiomes of both the animal gut and the microbiome of plants are known for their role in their host's nutrient uptake, protection against pathogens and abiotic stress as well as providing metabolic capacities (Sekirov *et al.*, 2010, Mitter *et al.*, 2013). Extensive efforts to characterize the plant microbiome, coupled with exciting advances in sequencing technologies and in computational techniques, have increased our knowledge of the diversity, its structure-function relationships and its composition. Plant microbiome composition is affected by various host-driven factors, including for instance the plant genotype, and by agricultural practices such as soil tillage, crop rotation, fertilization or pesticides application (Sessitsch & Mitter, 2014). Our scientific understanding regarding agriculture driven microbiome alterations in important crops like OSR is limited.

In response, the goals of this thesis were to expand our knowledge of the OSR associated bacterial microbiome under the impact of plough based conventional tillage *vs* conservation strip tillage practices. The work presented in Chapter 3, 4 & 5 was achieved by generating high-resolution 16S rRNA gene amplicon sequencing using the Illumina MiSeq platform and by using the bioinformatic tools Qiime and Usearch pipelines to analyse the amplicon sequence data. In Chapter 3, we first investigated the complex microbial communities' structure and composition residing in different microhabitats (*viz.*, bulk soil, rhizosphere, root and shoot) at plant maturity. The data showed that the root and shoot associated bacterial communities displayed markedly distinct profiles as a result of tillage practices. We observed a limited 'rhizosphere effect' in the root zone of WOSR at plant maturity stage. Based on the knowledge developed from this study a subsequent investigation into the rhizosphere microbiota establishment at different plant growth stages (*viz.*, vegetative, flowering and harvesting stage) was carried out (Chapter 4). The data suggest that the rhizosphere microbiota was similar to bulk soil at all growth stages

under CT, whereas the impact of ST became stronger at later growing stage. Chapter 5 further explored the niche differentiation of WOSR bacterial microbiome between the plant environments (*viz*., bulk soil, rhizosphere, root and shoot), at three plant developmental stages, under the influence of soil tillage. Our findings show that tillage and growth stages were significant determinants of bacterial community niche separation in different plant associated microhabitats. Moreover, overall WOSR microbiome study was found to be composed of *Kouleothrixaceae, Actinosynnemataceae, Bacillaceae, Bradyrhizobiaceae, Caulobacteraceae, Comamonadaceae, Flavobactreriaceae, Gaiellaceae, Hyphomicrobiaceae, Intrasporangiaceae, Methylophillaceae, Microbacteriaceae, Micrococcaceae, Nocardiaceae, Nocardioidaceae, Oxalobacteraceae, Pseudomonadaceae, Solirubrobacteraceae, Sphingomonadaceae, Aurantimonadaceae, Deinococcaceae, Desulfuromonadaceae, Trueperaceae, Alteromonadaceae, Micromonosporaceae, Sinobacteraceae, Steptomycetaceae, Xanthomonadaceae, Weeksellaceae, Brucellaceae, Enterobacteriaceae* and *Sanguibacteraceae* which were influenced by both plant growth stages as well as soil tillage practices. Chapter 6 was focused on investigating the impact of WOSR and wheat crop rotation, in combination with soil tillage, on prevalence of 2,4-DAPG producing bacteria in rhizosphere and root microhabitats of WOSR and wheat crops, at different plant growth stages. The study involved assessing the prevalence of 2,4-DAPG producing microbes in rhizosphere and root microhabitats of WOSR and wheat crops. To achieve this task, quantitative PCR technique was used to quantify the *PhlD* gene that is responsible for the production of 2,4-DAPG compound.

WOSR is often used as the break crop in cereal crop rotation. Therefore, it is important to study WOSR microbial diversity and its composition that is retained at plant maturity stage. As after harvesting of the crop, plant roots are left in soil until the next crop planting. During this time period the roots decompose and release root associated microbes into the soil. In this way plant associated microbes can be transferred from one crop cycle to the next (Sturz & Christie (1995) and Sturz *et al.* (1998)). However, while evaluating the significantly enriched OTUs as a result of tillage regimes, our results showed that bulk soil exhibited no significant difference in the OTU enrichment between both tillage practices. On the other hand, compartments such as the rhizosphere, root and shoot showed significant OTUs enrichment in each tillage regime (Table A3.3). Smith *et al.* (2016) reported a

difference in soil microbial community composition, function and soil nutrient profiling between conventional and conservation tillage practices. Furthermore, Dorr de Quadros *et al.* (2012) stated that overall soil microbial composition was different between different tillage practices. They also observed that anaerobic bacterial composition was higher in conservation tillage, whereas aerobic bacteria were enriched in conventional tillage system. However, in our case we did not observe any significant difference in bulk soil microbiomes between CT and ST practices. Possible reasons for this could be the fact that in our experimental field it was the very first time it had been tilled using a conservation strip tillage system. It may be the case that multiple seasons of conservation tillage are required before significant differences in the soil microbiome become apparent.

In this study, minor differences were observed in bacterial richness and abundance between the OSR rhizosphere and bulk soil at harvesting stage. Therefore, we hypothesised that a significant difference between the bulk soil and rhizosphere would exist, perhaps at earlier plant growth stage which is further reduced at later (harvest) growth stage under the influence of soil tillage practices. As a result we studied rhizospheric bacterial establishment at different OSR plant growth stages under the influence of CT and ST practices (Chapter 4). Our results did not support our hypothesis as we observed no significant difference between the OSR rhizosphere and bulk soil at either the flowering or vegetative stages. Bulgarelli *et al.* (2012) and Lundberg *et al.* (2012) reported significant similarity of rhizosphere and bulk soil microbiota in *A. thaliana* (which is from the same botanical family as OSR) under different soil types. Rhizosphere microbiota is influenced by root exudation pattern, and that is affected by many soil environmental factors such as soil texture, pH, moisture, temperature and nutrient status of the soil (Haldar & Sengupta, 2015). Therefore, the discrepancy observed in OSR rhizosphere microbiota under ST at harvesting stage, would perhaps be driven by soil factors, as under CT and all other growth stages rhizosphere effect was negligible, though the WOSR plant genotype was same.

Moreover, this study revealed that the proportions of some WOSR associated bacterial communities were changing over the plants life cycle viz., vegetative, flowering and harvesting stages. Philippot *et al.* (2013) suggested that the rhizosphere microbiome is regulated by the quantity and composition of rhizodeposits, which in-turn is influenced by a variety of biotic and abiotic factors

and vary in time and space with respect to the position on the root. The structural and functional diversities of rhizosphere microbial communities of a wide variety of plants, including *Arabidopsis*, maize, pea, wheat and sugar beet are shaped by plant developmental stages. Chaparro *et al.* (2013) demonstrated a strong correlation between compounds released from the roots at different stages of plant development and the expression of microbial genes involved in metabolism of specific compounds. Metatranscriptomic analysis of the rhizosphere microbiome of *A. thaliana* (genotype Pna-10) revealed that eighty-one unique transcripts were significantly expressed at different stages of plant development (Chaparro *et al.*, 2013). It was concluded from these studies that the blend of compounds and phytochemicals in the root-exudates were differentially produced at distinct stages of plant development that influenced rhizobiota community structure and diversity. This study should be extended in the future to investigate the signal molecules (metabolomics) used by the plant/microbes to communicate under both tillage practices. This in turn will enhance the understanding of the factors that drive WOSR plant-microbe interactions under CT and ST at different plant growth stages.

Based on the results of the rhizosphere microbiome dynamics, we were motivated to study soil tillage driven niche differentiation in the rhizosphere and endosphere at three different OSR plant growth stages (Chapter 5). However, analysis of the endosphere microbiome proved to be difficult as the primers (341F - 785R, covers V3 and V4 hypervariable regions in 16S rRNA gene) used for the 16S amplicon sequencing in chapter 3 were not capable of discriminating between the microbial 16S and plant derived mitochondria and chloroplast sequences (Klindworth *et al.*, 2013). This primer pair (341F - 785R) has been the preferred target of the 16S rRNA in studying soil and rhizosphere assemblages (Hiergeist *et al.*, 2016). The selection of a suitable primer pair was challenging because of the high homology between bacterial 16S rRNA, chloroplast 16S rRNA and mitochondria 18S rRNA. Currently, three general methods exist to reduce the impact of these contaminating sequences: (a) adaptation of existing RNA extraction protocols to reduce co-extraction of organellar RNA (Lutz *et al.*, 2011) or post-extraction separation of host RNA from microbial RNA based on differences in CpG methylation density (Feehery *et al.*, 2013), (b) the development of blocking primers to block and/or reduce amplification of sequences originating from a eukaryotic host, such as peptide nucleic acid-mediated PCR clamping (Lundberg *et al.*, 2013) and

suicide polymerase endonuclease restriction (SuPER) (Green & Minz, 2005), and (c) the use of specific mismatch primers during PCR amplification (Chelius & Triplett, 2001).

The preferred or most utilized technique is to use specific mismatch primers, which amplify bacterial 16S rRNA gene sequences while simultaneously avoiding the amplification of chloroplast 16S rRNA gene sequences. Chelius & Triplett (2001) developed the first mismatch primer (799F), with a primer design which centred around two base pair mismatches at positions 798–799 and two additional base pair mismatches at positions 783 and 784 in the chloroplast RNA. The primer pair 799F-1193R has been used with varying success in several plant systems (Bulgarelli *et al.*, 2012, Bodenhausen *et al.*, 2013, Shade *et al.*, 2013). Therefore this primer pair was selected for an in-depth study of WOSR microbiome which possesses incorporated sequence mismatch to reduce co-amplification of chloroplast rRNA. It also amplifies a mitochondrial product of ~800 bp and bacterial product of ~400 bp. As a result, bacterial 16S rRNA product can be easily separated and isolated from the chloroplast and mitochrondrial derived PCR products on the agarose gel. Thus, in Chapter 5, the 799F-1193R primer pair was used for the amplicon sequencing and it covers the V5 to V7 hypervariable region of 16S rRNA gene.

While comparing the results of Chapter 3 and Chapter 5, we observed the difference in phylum distribution of harvesting stage samples (Figure 3.1 & 5.4). The two primer sets used in these separate studies covered two different hypervariable regions of the 16S rRNA gene, and exhibited different bacterial enrichment in root and shoot compartments even though the samples were same. For instance, 341F - 785R primers showed the dominance of bacterial members at phylum level *Proteobacteria, Actinobacteria, Bacteroidetes* and *Firmicutes,* whereas 799F - 1193R recovered the predominant phyla *Proteobacteria, Actinobacteria, Chloroflexi, Bacteroidetes* and *TM7.* These lead to the distinct outcomes of tillage dependent effect in root and shoot compartments. For example, in the phylum distribution graph 341F - 785R primers showed substantial difference between tillage regimes in the root compartment (Chapter 3; Figure 3.1), whereas the 799F - 1193R primers showed significant difference in the in shoot compartment (Chapter 5; Figure 5.4).

Baker *et al.* (2003) provided a detailed review regarding the bias in phylogenetic analysis that can be introduced through differential amplification

caused by differences in the efficiency of primer binding, interference by sequences flanking primer regions (Hansen et al., 1998) and differences in the kinetics of the PCR reaction (Reysenbach *et al.*, 1992, Brunk & Eis, 1998). As a consequence, many, if not all, 16S rRNA libraries will not be totally representative of microbial communities, especially on a quantitative level (Reysenbach *et al.*, 1992, Farrelly *et al.*, 1995). Furthermore, Klindworth *et al.* (2013) reported that the primer pair 341F - 785R although showing higher overall coverage, failed to detect seven bacterial phyla including Hyd24-12, GOUTA4, *Armatimonadetes, Chloroflexi*, and Candidate divisions OP11, WS6 and TM7. On the other hand, in our data the primer pair 799F – 1193R showed the presence of *Chloroflexi* and TM7 in WOSR microbiome. Therefore, alternative PCR-independent approaches such as shotgun based metagenomic studies, could be beneficial to investigate bacterial communities in a given environment if time and funds are available that would allow overcoming the limitation of PCR-dependent method.

The impact of tillage practices on WOSR microbiome establishment was characterised in Chapters 3, 4 & 5. It was then proposed to explore the long-term impact of tillage practices, along with crop rotation, on the prevalence of 2,4-DAPG producing microbial spp. that thrive in rhizosphere and root of WOSR and wheat crops, at three plant growth stages. The antibiotic 2,4-DAPG has a broad spectrum of activity and is especially active against the take-all pathogen (Kwak & Weller, 2013). This antibiotic biosynthetic locus includes the five gene operon *PhlACBDE*, wherein the *PhlD* gene is primarily involved in biosynthesis of phloroglucinol. Therefore, in this current work the *PhlD* gene copy number was studied to estimate the prevalence of this gene.

In general, the results of this thesis chapter showed that the prevalence of the *PhlD*$^{+}$ was considerably higher under ST compared to the CT regime under both crop rotation and monoculture practices. This result is also supported by the outcomes of the previous thesis Chapters 3, 4 & 5, that revealed the significant enrichment of *Pseudomonadaceae* family under ST while studying the WOSR microbiome. The family distribution graphs in Chapter 3 (Figure 3.5), Chapter 4 (Figure 4.5) and Chapter 5 (Figure 5.5), were calculated based on bacterial families that present >1 % average relative abundance of sequence reads. In Chapter 5, the bacterial members from *Pseudomonadaceae* family were observed in WOSR root compartment, 12.5 % and 1.3 % in ST and CT respectively at flowering stage,

whereas at harvesting stage, 10.7 % in ST and 3.8 % in CT. While studying the WOSR rhizosphere microbiome in Chapter 4, the average relative abundance of *Pseudomonadaceae* family was only observed at harvesting stage with 3.4 % in ST and 0.6 % in CT regime.

Recently, Robertson-Albertyn *et al.* (2017) reported that root hairs play a critical role in resource exchanges between plants and the rhizosphere, and they can act as a determinant of the bacterial colonisation at the root–soil interface. Interestingly, in grasses, root hairs also define an evolutionarily conserved site for bacterial colonization. For instance, the beneficial bacterium *Pseudomonas* spp. DSMZ 13134 efficiently colonizes the root hairs of either soil- or quartz sand-grown barley seedlings (Buddrus-Schiemann *et al.*, 2010). Prieto *et al.* (2011) studied the root hairs' role in endophytic bacterial colonisation of olive roots by using the two biocontrol strains PICF7 and PICP2 of *Pseudomonas* spp. They observed that colonization of root hairs appeared to be a highly specific event, and only a very low number of root hairs were effectively colonized by introduced bacteria. The authors also suggested that many environmental factors can affect the number, anatomy, development, and physiology of root hairs, as well as colonization competence and biocontrol effectiveness of *Pseudomonas* spp. which perhaps greatly influenced by root hair's fitness. Furthermore, edaphic factors such as soil structure, texture, porosity, nutrient pool, temperature, moisture, and pH can considerably influence the root growth, root hair structure and composition of rhizodeposition (Prieto *et al.*, 2011, Guan *et al.*, 2015, Thorup-Kristensen & Kirkegaard, 2016). These edaphic soil qualities are influenced by the tillage practices (Vian *et al.*, 2009, Guan *et al.*, 2015). Therefore, we hypothesised that perhaps CT and ST system have influenced the WOSR and wheat root growth, and associated changes in root hair morphology that lead to the differential colonisation of *Pseudomonas* spp. in rhizosphere and root microhabitats.

This research has shown that agriculture management practices are major driving factors of the WOSR microbiome in the rhizosphere and endosphere environments. It advances the understanding of WOSR bacterial microbiome niche differentiation over the plants life cycle. Plants and associated microbiota evolved together and have developed a mutualistic relationship where both partners benefit from the association. However, intensive agricultural practices have unintentionally

affected this association, resulting in the loss of key beneficial members of the crop microbiome.

The rational manipulation of the plant-microbiome traits, through agricultural management practices, could be a possible solution to help in enhancing the sustainability of crop production, through promoting the development of beneficial plant growth promoting and disease suppressing members of the rhizosphere and endosphere microbiomes. However, more detailed and extensive research is essential to fill the gaps in our knowledge regarding the plant-soil-microbial interactions as a multi-dimensional network between the plant, its microbiome, the diverse environmental conditions, and the geochemical context of the soil. This will further help in cracking the so called ‘black box’ of microbiome ecology and help to unlock the true potential of these fascinating microorganisms for eco-friendly agriculture. Strategic approaches to effectively recognise the role of microbes in above- and below-ground processes due to agriculture management systems should not only be concentrated on plant effects, but also on the microbiome itself, its functionality, the artificial inoculation of microbes, and would try to consider the plant as well as microbial perspective.

7.1 **Future Work**

Based on this current research outcome, a solid conclusion, to change the WOSR cultivation practices from CT to ST cannot be proposed due to the constraints of time and resources. However, to sensibly deploy the WOSR microbiome through soil tillage management, this would require further investigations.

- **Agronomy prospect**

to determine the tillage impact on WOSR microbiome (bacterial & fungal) over the successive years along with the crop rotation practices, and enrich this information with functional studies (either metagenomics, metatranscriptomics or metabolomics) to harness the habitat-specific microbial traits.

- **Breeding prospect**

to explore the complete characterization of the phytobiome associated with WOSR crop varieties grown under different environmental and climatic conditions for the characterization of WOSR core microbiome. Consider the plant microbiome aspect while breeding the next generation WOSR cultivars to enhance the mutualistic interactions with beneficial microbes and maintain this beneficial microbial pool in the soil habitat though agricultural practices.

- **Industrial prospect**

to isolate highly abundant microbial OTUs for industrial application processes. Examine the effect of tillage practices on the WOSR seed microbiome and determine its possible implications on edible rape oil/biodiesel quality.

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Appendix A3

Table A3.1 Physical and chemical characterisation of the soil substrates

Mineral content ($g \cdot kg^{-1}$)	Conventional tillage (CT)	Conservation strip tillage (ST)
Total carbon	2.93	2.95
Total nitrogen	0.27	0.27
Soil organic matter	5.72	5.79
Soil organic carbon	2.52	2.55
C/N ratio	11.93	12.09
pH	6.09	6.15
Classification	Sandy loam	Sandy loam

Table A3.2 Proportion of reads with and without plant derived of WOSR samples

Sample ID	Compartment type	Reads with plant derived reads	Reads without plant derived reads	Proportion of without plant derived reads (%)
Bn 25	Shoot - ST	73043	38699	52.98
Bn 26	Root - ST	42615	23886	56.05
Bn 27	Rhizosphere - ST	65236	65113	99.81
Bn 28	Bulk soil - ST	48100	47972	99.73
Bn 29	Shoot - ST	69672	35422	50.84
Bn 30	Root - ST	42791	23984	56.05
Bn 31	Rhizosphere - ST	34683	34599	99.76
Bn 32	Bulk soil - ST	32385	32323	99.81
Bn 33	Shoot - ST	87433	38828	44.41
Bn 34	Root - ST	47772	26879	56.27
Bn 35	Rhizosphere - ST	36906	36782	99.66
Bn 36	Bulk soil - ST	15215	15178	99.76
Bn 61	Shoot - CT	32521	18293	56.25
Bn 62	Root - CT	69757	31552	45.23
Bn 63	Rhizosphere - CT	36306	36214	99.75
Bn 64	Bulk soil - CT	39568	39461	99.73
Bn 65	Shoot - CT	55541	30048	54.10
Bn 66	Root - CT	22487	9765	43.43
Bn 67	Rhizosphere - CT	6218	6191	99.57
Bn 68	Bulk soil - CT	14735	14701	99.77
Bn 69	Shoot - CT	45801	28416	62.04
Bn 70	Root - CT	31990	15278	47.76
Bn 71	Rhizosphere - CT	17333	17303	99.83
Bn 72	Bulk soil - CT	24418	24343	99.69
	Total	992526	691230	----
No. of OTUs **with** Plant derived				2182
No. of OTUS **without** Plant derived				2161
Total useful reads in percentage				69.64%
Max, Min, and Mean no. of reads in dataset				
Max				28801.25
Min				65113
Mean				6191

*ST means conservation strip tillage and CT means conventional tillage.

Table A3.3 Significantly enriched OTUs in comparison to tillage practices (CT *vs* ST) in bulk soil and plant microhabitat zones

Sample type	Enriched OTUs	Taxonomy of enriched OTUs
Bulk soil - CT	0	
Bulk soil - ST	0	
Rhizosphere - CT	5	p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae
		p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales f__Xanthomonadaceae
		p__Firmicutes c__Bacilli o__Bacillales f__Bacillaceae
		p__Bacteroidetes c__Sphingobacteriia o__Sphingobacteriales f__Sphingobacteriaceae
		p__Bacteroidetes c__Sphingobacteriia o__Sphingobacteriales f__Sphingobacteriacea
Rhizosphere - ST	9	p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Hyphomicrobiaceae
		p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Hyphomicrobiaceae
		p__Proteobacteria c__Alphaproteobacteria o__Sphingomonadales f__Sphingomonadaceae
		p__Proteobacteria c__Deltaproteobacteria o__Myxococcales f__
		p__Verrucomicrobia c__Opitutae o__Opitutales f__Opitutaceae
		p__Verrucomicrobia c__[Pedosphaerae] o__[Pedosphaerales] f__auto67_4W
		p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Nocardiaceae
		p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Nocardioidaceae
		p__Nitrospirae c__Nitrospira o__Nitrospirales f__Nitrospiraceae
Root - CT	13	p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Hyphomicrobiaceae
		p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Hyphomicrobiaceae
		p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales f__Xanthomonadaceae
		p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales f__Xanthomonadaceae
		p__Proteobacteria c__Gammaproteobacteria o__Xanthomonadales f__Sinobacteraceae
		p__Proteobacteria c__Deltaproteobacteria o__Myxococcales f__Haliangiaceae
		p__Proteobacteria c__Deltaproteobacteria o__Myxococcales f__
		p__Chloroflexi c__Ellin6529 o__ f__
		p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Thermomonosporaceae
		p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Micromonosporaceae

		p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Nocardiaceae
		p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Sanguibacteraceae
		p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Thermomonosporaceae
Root - ST	10	p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae
		p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae
		p__Proteobacteria c__Gammaproteobacteria o__Enterobacteriales f__Enterobacteriaceae
		p__Proteobacteria c__Gammaproteobacteria o__Pseudomonadales f__Pseudomonadaceae
		p__Proteobacteria c__Alphaproteobacteria o__Caulobacterales f__Caulobacteraceae
		p__Firmicutes c__Bacilli o__Bacillales f__Bacillaceae
		p__Firmicutes c__Bacilli o__Bacillales f__Bacillaceae
		p__Bacteroidetes c__Flavobacteriia o__Flavobacteriales f__Flavobacteriaceae
		p__Bacteroidetes c__Flavobacteriia o__Flavobacteriales f__[Weeksellaceae]
		p__Bacteroidetes c__Sphingobacteriia o__Sphingobacteriales f__Sphingobacteriaceae
Shoot - CT	9	p__Proteobacteria c__Betaproteobacteria o__Methylophilales f__Methylophilaceae
		p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Comamonadaceae
		p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Brucellaceae
		p__Firmicutes c__Bacilli o__Bacillales f__Paenibacillaceae
		p__Bacteroidetes c__Flavobacteriia o__Flavobacteriales f__[Weeksellaceae]
		p__Bacteroidetes c__[Saprospirae] o__[Saprospirales] f__Chitinophagaceae
		p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Nocardiaceae
		p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Streptomycetaceae
Shoot - ST	1	p__TM7 c__TM7-3 o__ f__

*Right, number of significantly enriched OTUs retrieved from a moderated estimation of fold change and pair-wise Wald test (FDR, $P < 0.05$) between tillage practices (CT *vs* ST) of WOSR prokaryotic microbiota profiles. Left, taxonomy of frequently enriched OTUs. (CT) = conventional tillage, (ST) = conservation strip tillage, (p) = phylum, (c) = class, (o) = order, (f) = family.

Table A3.4 Overview of OTU data for the microbiota of WOSR at harvesting stage

	Complete Microbiome (Average No. of OTUs per sample)	Core Microbiome (No. of OTUs common to all six samples)	Dominant OTUs in Core Microbiome (No. of common OTUs ≥1% abundance)
Bulk soil	969 ± 48 (899-1036)	375	15 [8-12]
Rhizosphere	926 ± 114 (800-1068)	292	22 [14-17]
Root	438 ± 115 (334-577)	143	21 [15]
Shoot	150 ± 16 (133-180)	68	25 [15]

Data is based on the rarefied data set. Numbers in ( ) brackets represents the ranges. Numbers in [ ] represent the number of taxa present at the family level.

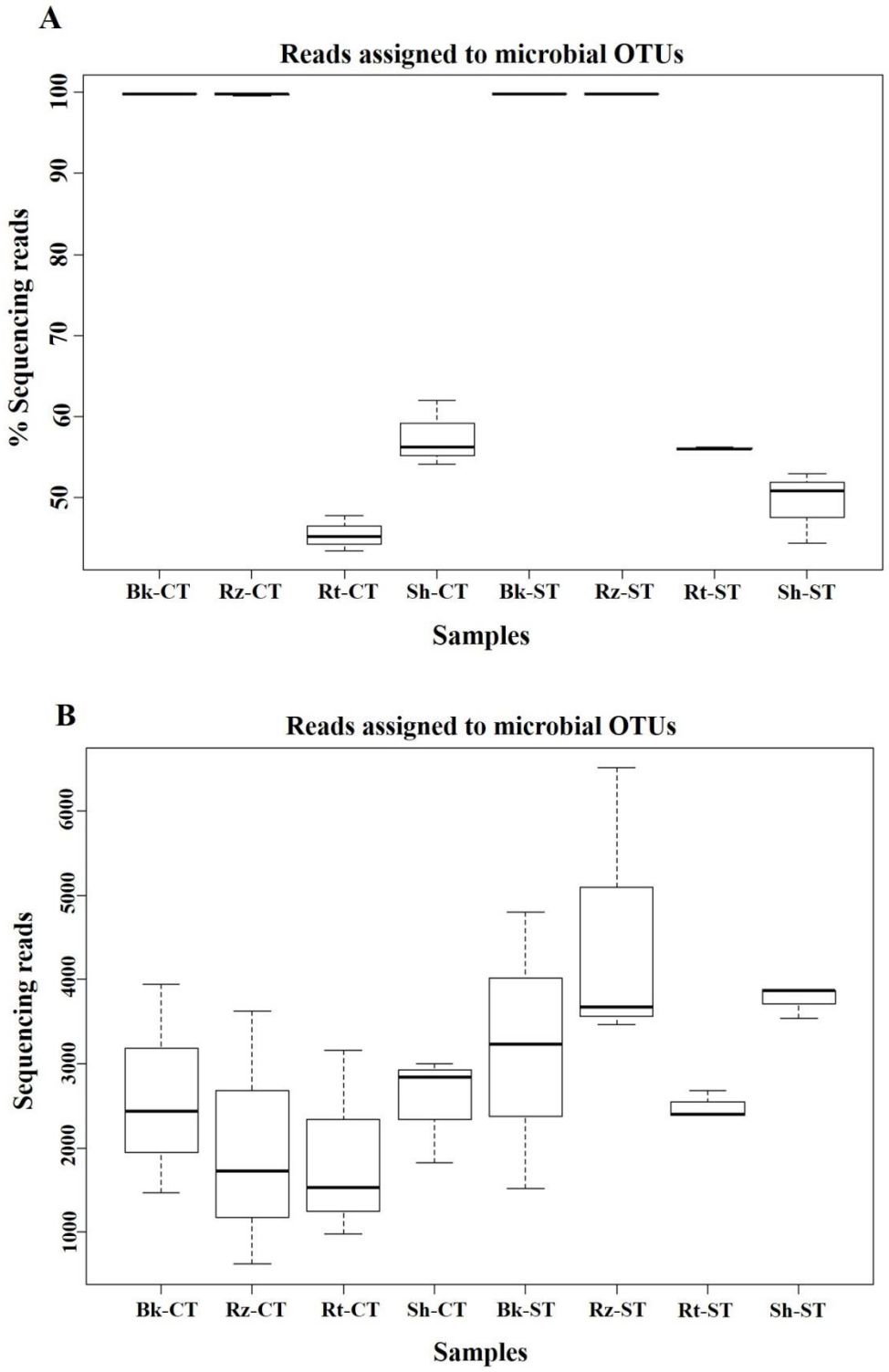


Figure A3.1 (A) Proportion of microbial-derived OTUs across compartments. X axis refers the sample type and Y axis refers the % reads assigned to microbial- derived OTUs. (B) Number of average reads per sample type. X axis refers the sample type and Y axis refers the number of reads assigned to microbial-derived OTUs.

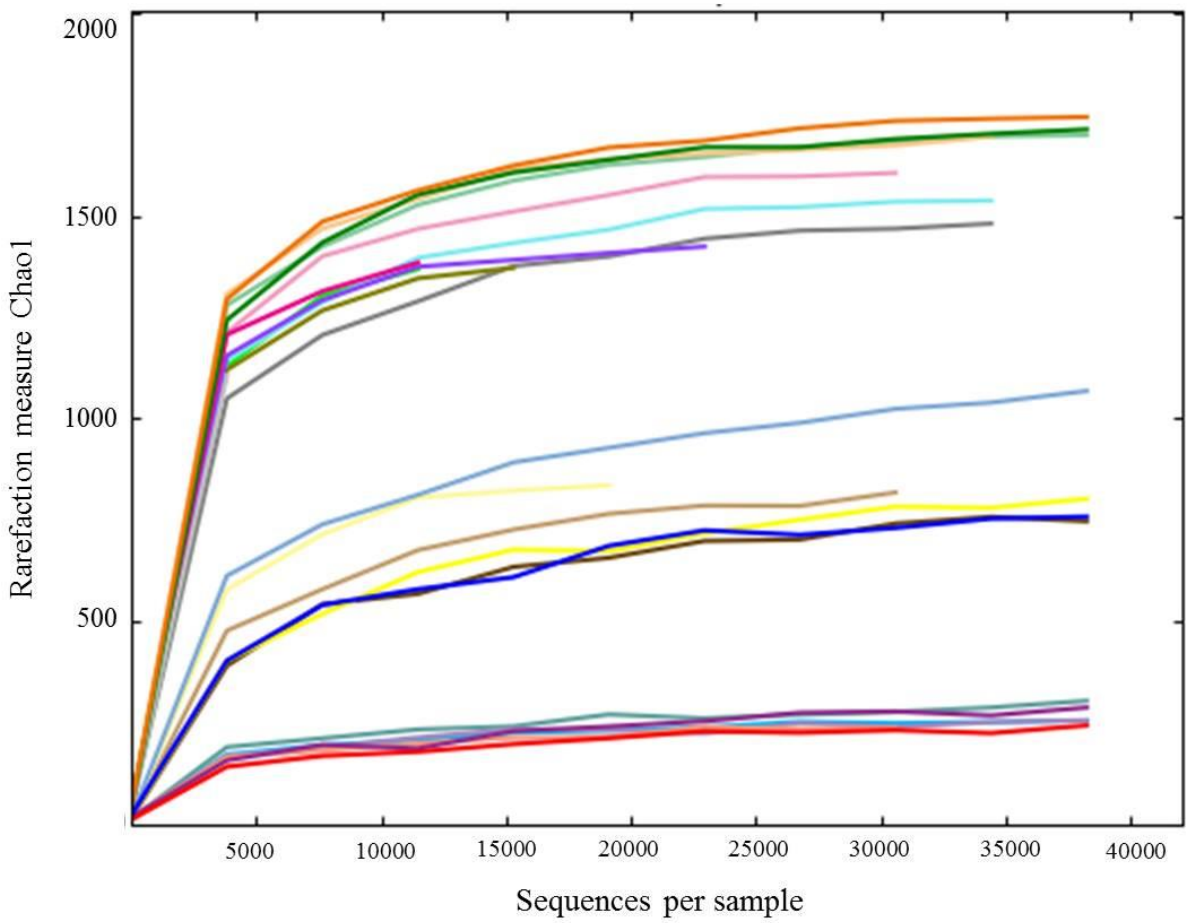


Figure A3.2 Rarefaction curves of individual samples from WOSR, generated in qiime.

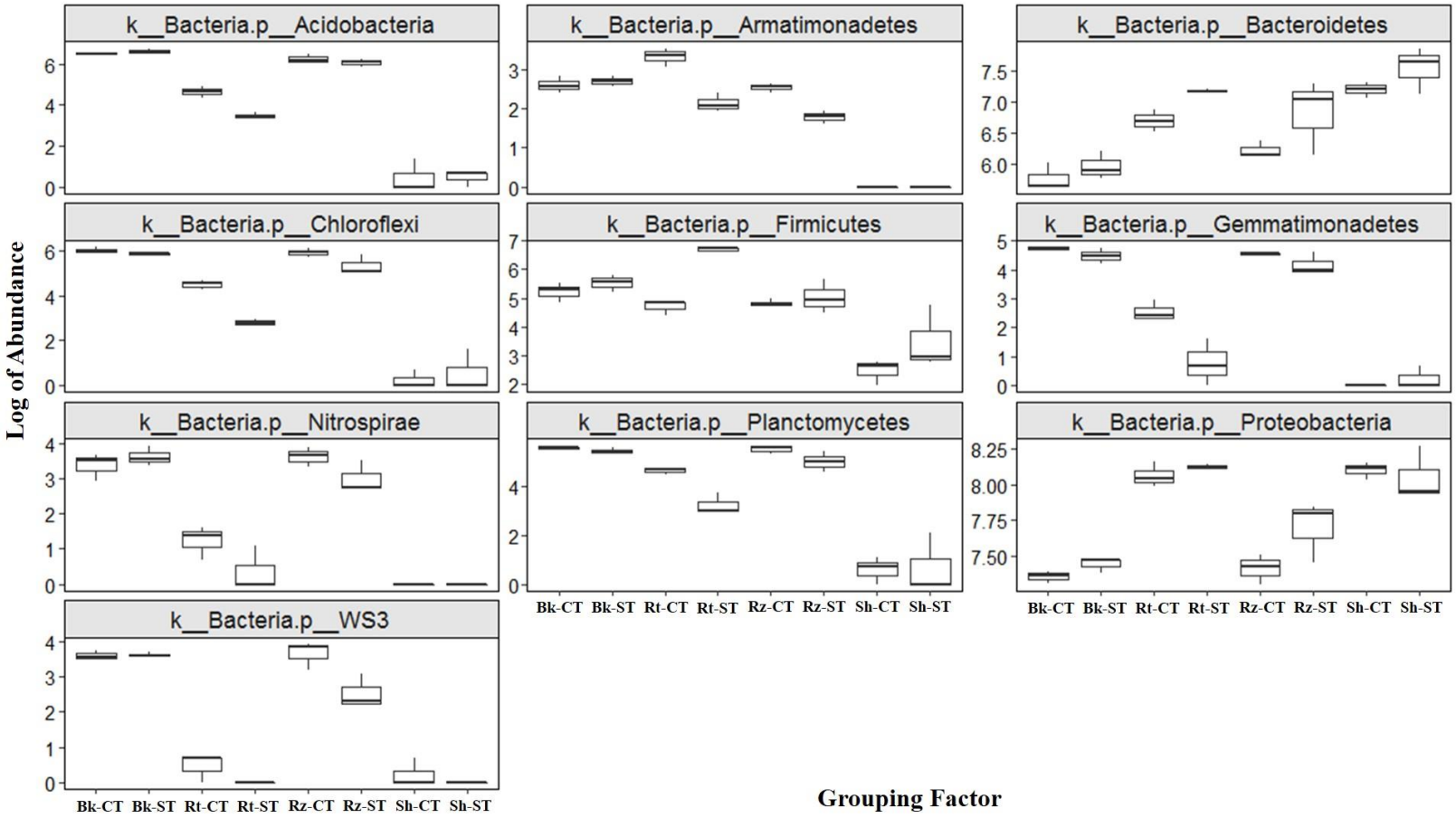


Figure A3.3 Differential composition of WOSR bacterial microbiota at phylum level (ANCOM, $P < 0.05$, FDR corrected).

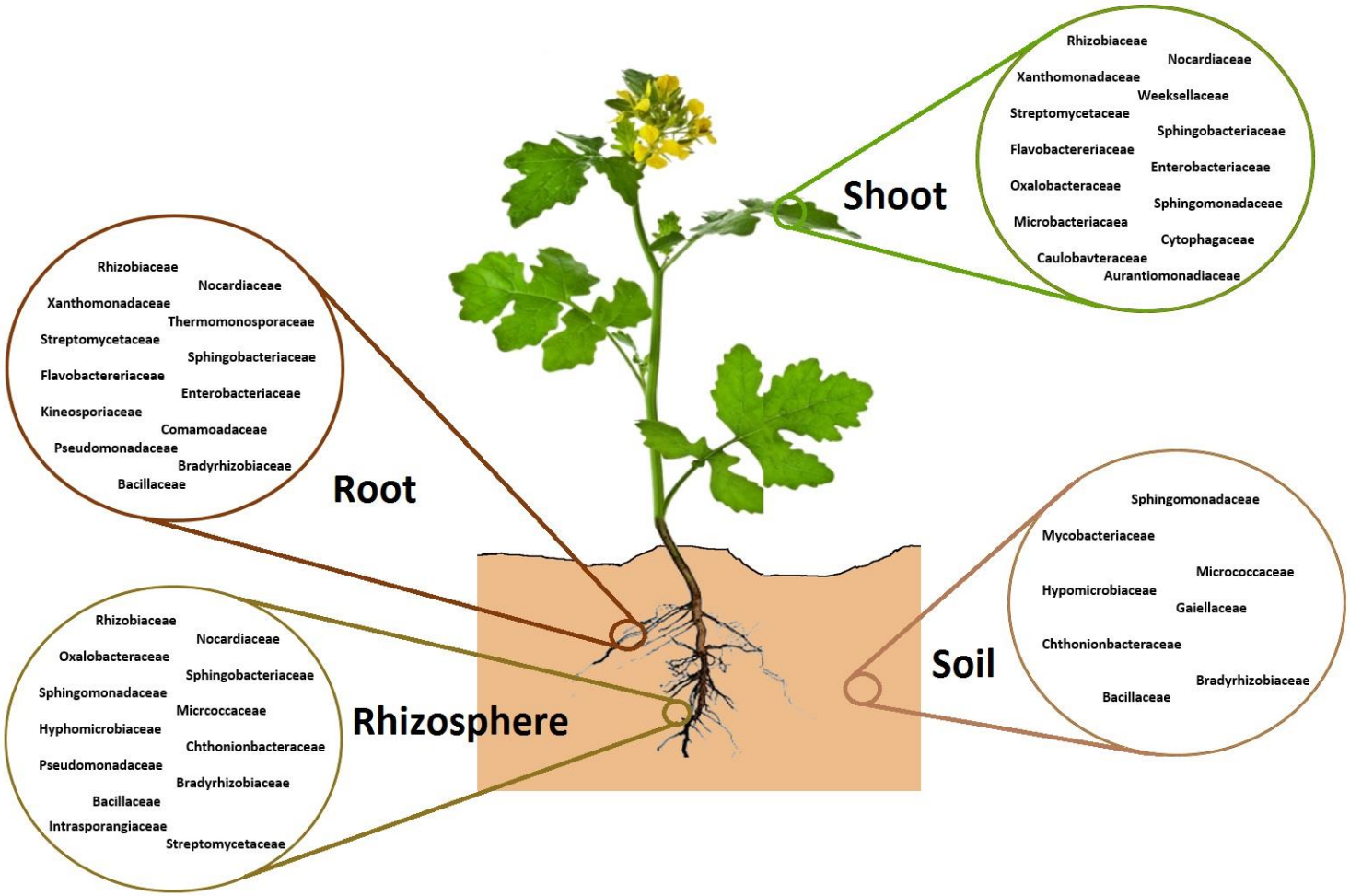


Figure A3.4 The conserved dominant microbiota of WOSR (*Brassica napus* var. compass) at harvesting stage. The conserved dominant microbiota is defined as those OTUs found in all six samples of a microhabitat, at greater than 1 % relative abundance (e.g. the core shoot microbiota are those OTUs found in all six samples of shoot material at greater than 1 % relative abundance)

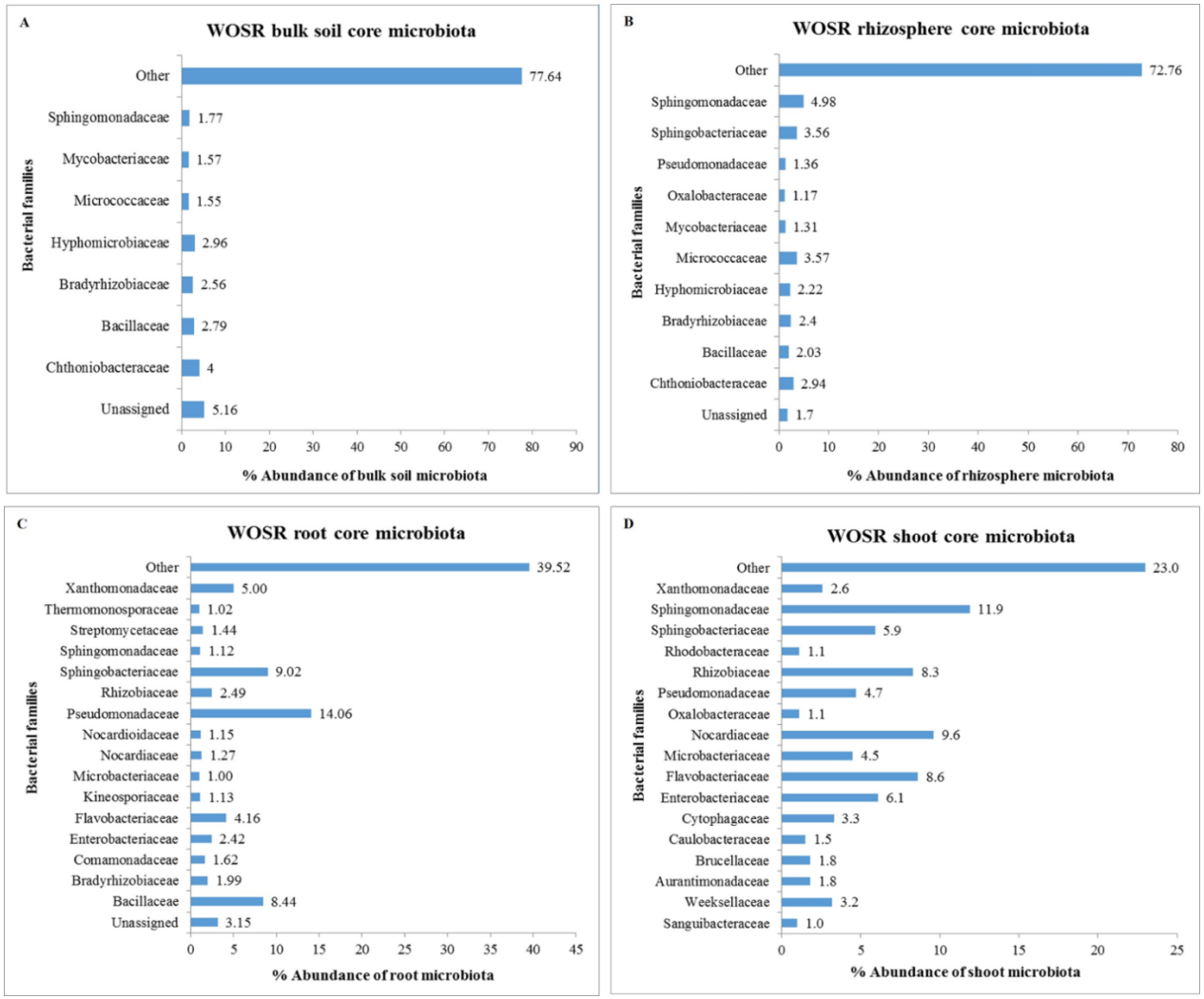
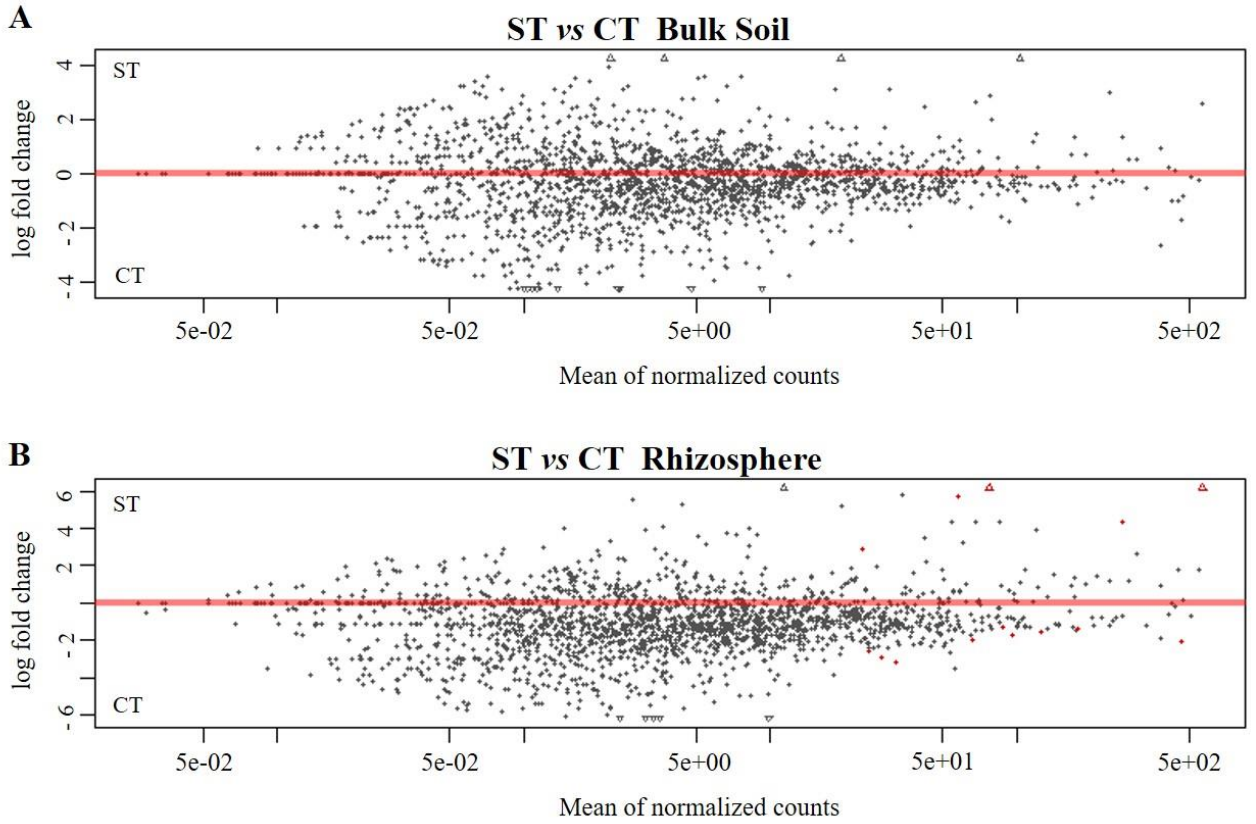


Figure A3.5 The conserved microbiota of the WOSR in bulk soil (A), rhizosphere (B), root (C) and shoot (D) compartments at harvesting stage based on the rarefied data set.


A
ST vs CT Bulk Soil
ST
CT
log fold change
4
2
0
-2
-4
5e-02
5e-02
5e+00
5e+01
5e+02
Mean of normalized counts
B
ST vs CT Rhizosphere
ST
CT
log fold change
6
4
2
-2
-6
5e-02
5e-02
5e+00
5e+01
5e+02
Mean of normalized counts

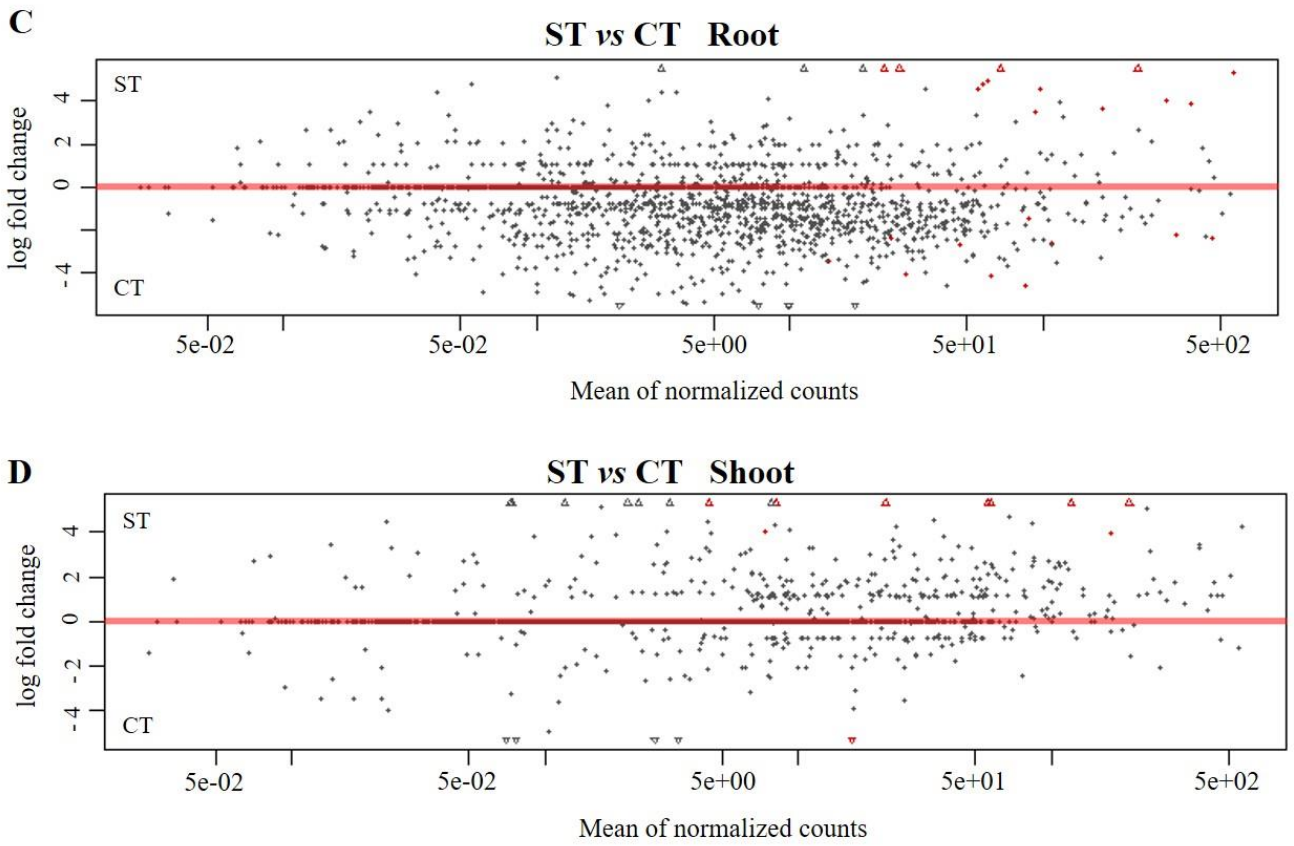


Figure A3.6 Pair-wise comparisons of the tillage practices; CT and ST under individual compartments; (A) bulk soil, (B) rhizosphere, (C) root and (D) shoot for enriched OTUs. In each plot, the shapes depict individual OTUs whose position on the x-axis reflect their abundance (normalised counts) and the position on the y-axis the fold change in the indicated comparison. The red colour depicts OTUs whose abundance is significantly different in the indicated comparisons (Wald test, $P < 0.05$, FDR corrected).

Appendix A5

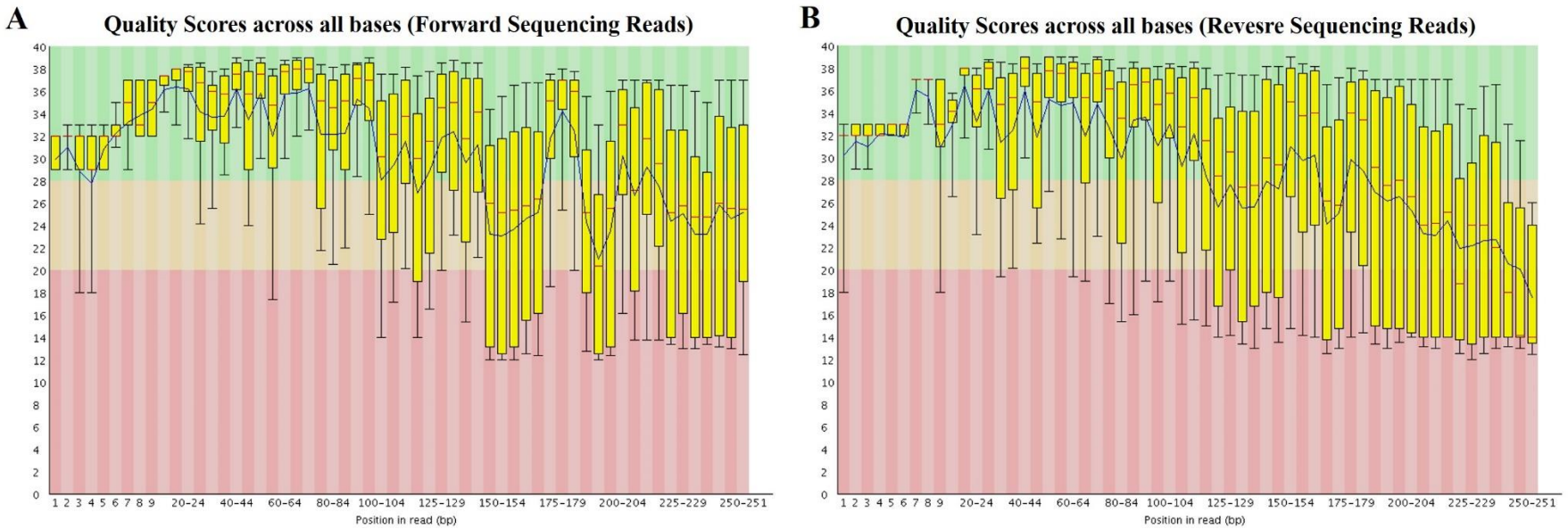


Figure A5.1 Graphs of quality score across all bases of (A) forward sequence reads and (B) reverse sequence reads.

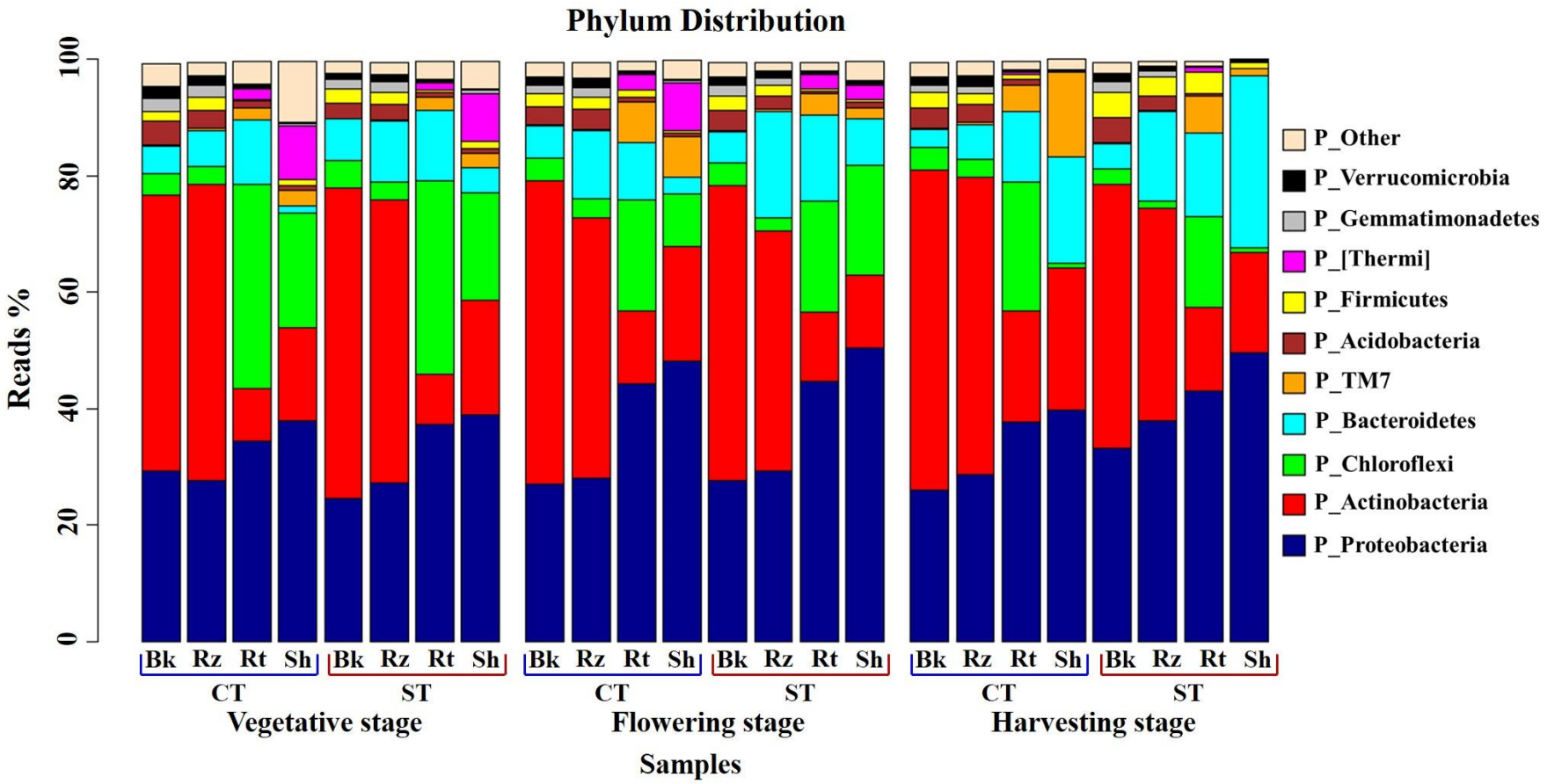
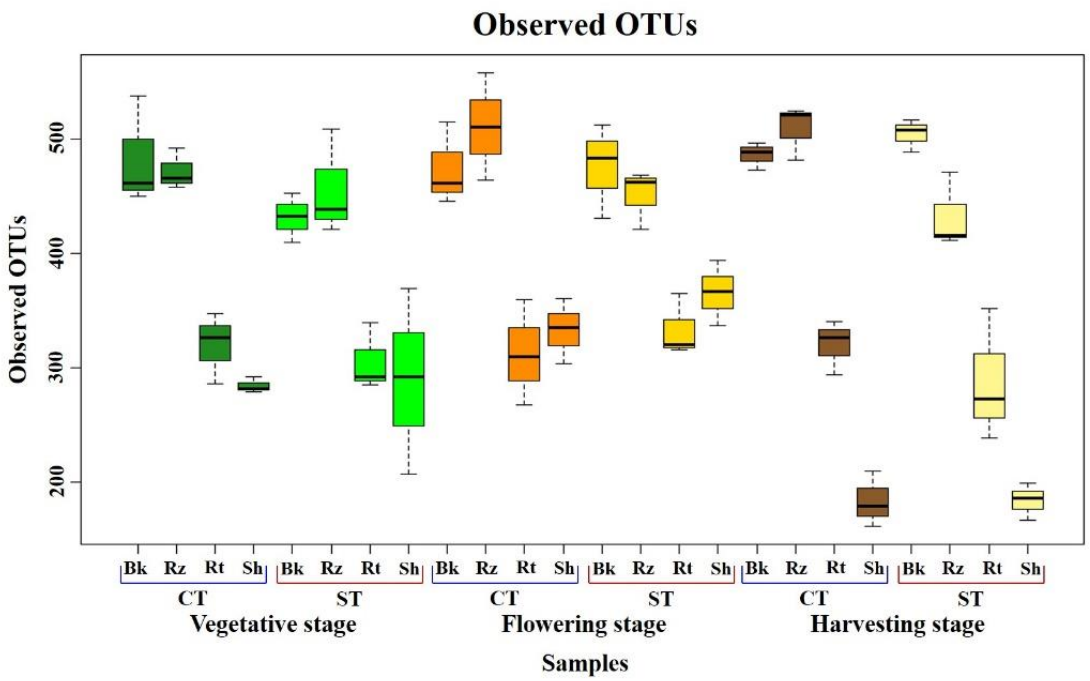
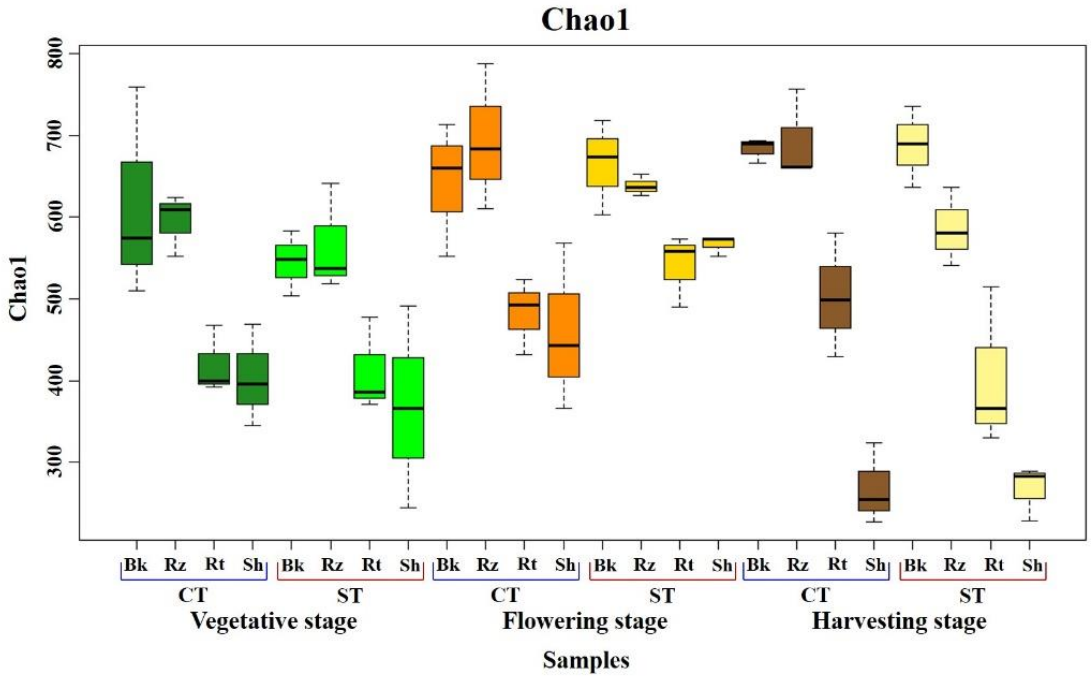


Figure A5.2 Phylum distribution of the OTUs (Reverse reads). Average relative abundance (% of sequencing reads) of 10 most abundant prokaryotic phyla associated with soil, rhizosphere, root and shoot microhabitat zones of WOSR under conventional tillage (CT) and conservation strip tillage (ST), are displayed in different colors. For each sample type, the number of replicates are n = 3. (Bk) bulk soil, (Rz) rhizosphere, (Rt) root, (Sh) shoot


Observed OTUs
Observed OTUs
200
300
400
500
Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh
CT ST CT ST CT ST
Vegetative stage
Flowering stage
Harvesting stage
Samples


Chao1
Chao1
300
400
500
600
700
800
Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh Bk Rz Rt Sh
CT ST CT ST CT ST
Vegetative stage
Flowering stage
Harvesting stage
Samples

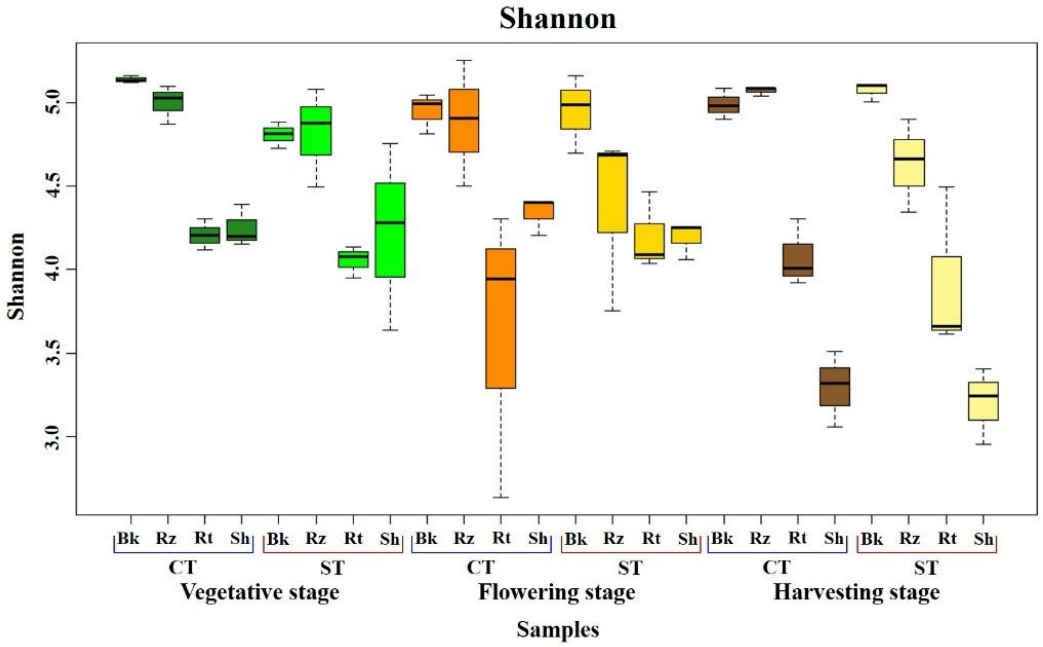


Figure A5.3 Alpha diversity analysis (Reverse reads). Variation patterns of alpha diversities of the bacterial communities associated with bulk soil, rhizosphere root and shoot microhabitats under two tillage practices; CT and ST at three plant growth stages. The alpha diversity estimates; Total number of observed OTUs, Chao1 estimator and Shannon's diversity are displayed in A, B and C respectively. (Bk) bulk soil, (Rz) rhizosphere, (Rt) root, (Sh) shoot, (CT) conventional tillage, (ST) conservation strip tillage. Sequencing reads of soil samples were rarefied at an even sequencing depth 6,781 reads/sample prior the analysis. Statistical analysis of alpha diversity are displayed in supplementary excel file WS- 1 to 4. Data are represented by three replicates from each stage.

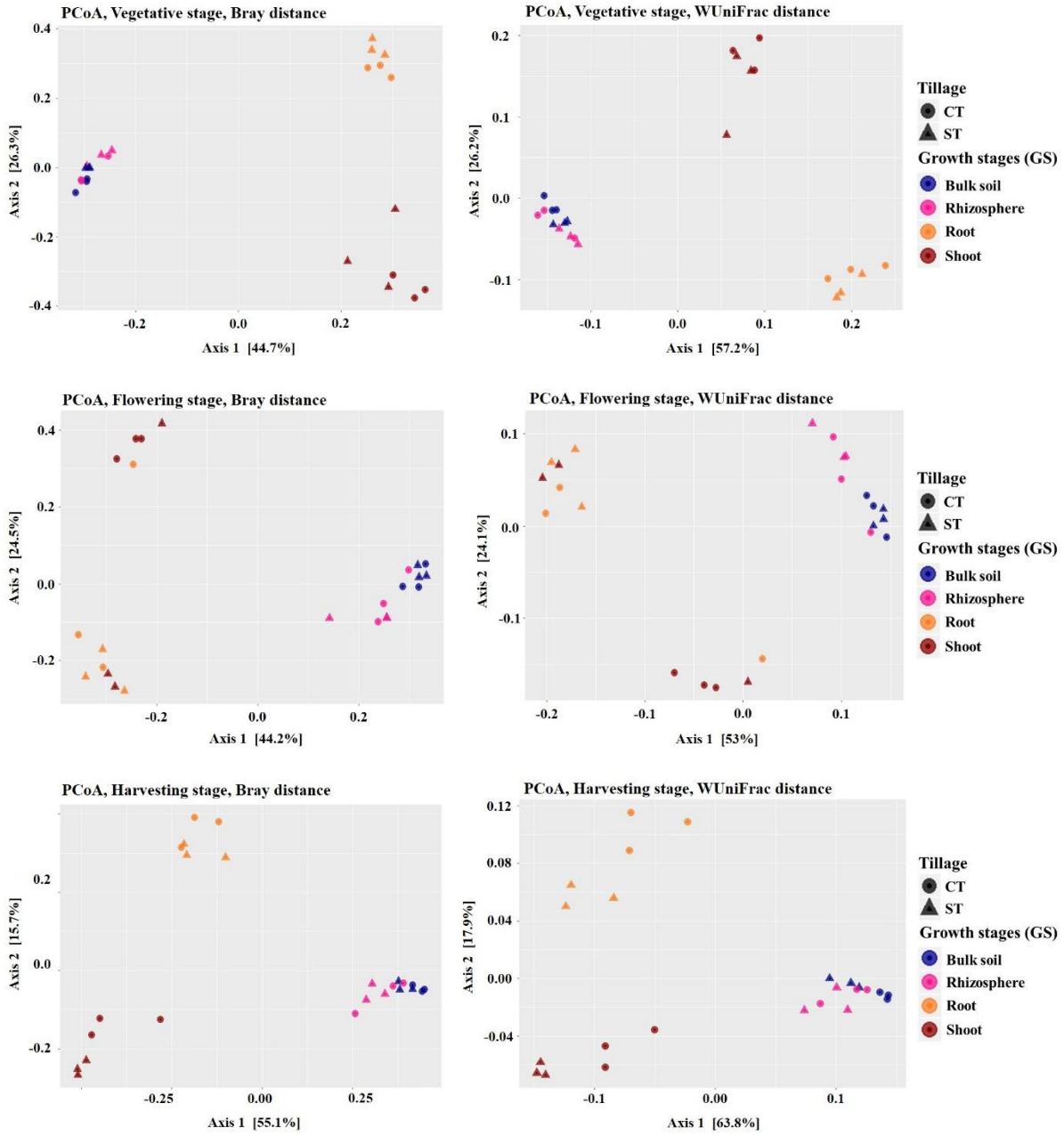


Figure A5.4 Beta diversity analysis (Reverse reads). Bacterial community structure of bulk soil, rhizosphere, root and shoot under CT and ST tillage practices at vegetative (A), flowering (B) and harvesting stages (C). Principal Coordinate Analysis (PCoA) based on Bray-Curtis (BC) and Weighted UniFrac (WUF) distances calculated using counts per million transformed OTU abundances. In both panels, colours define the compartments, while shapes depict the indicated tillage practices. Statistical results of beta diversity are displayed in Supplementary Excel File WS-2.

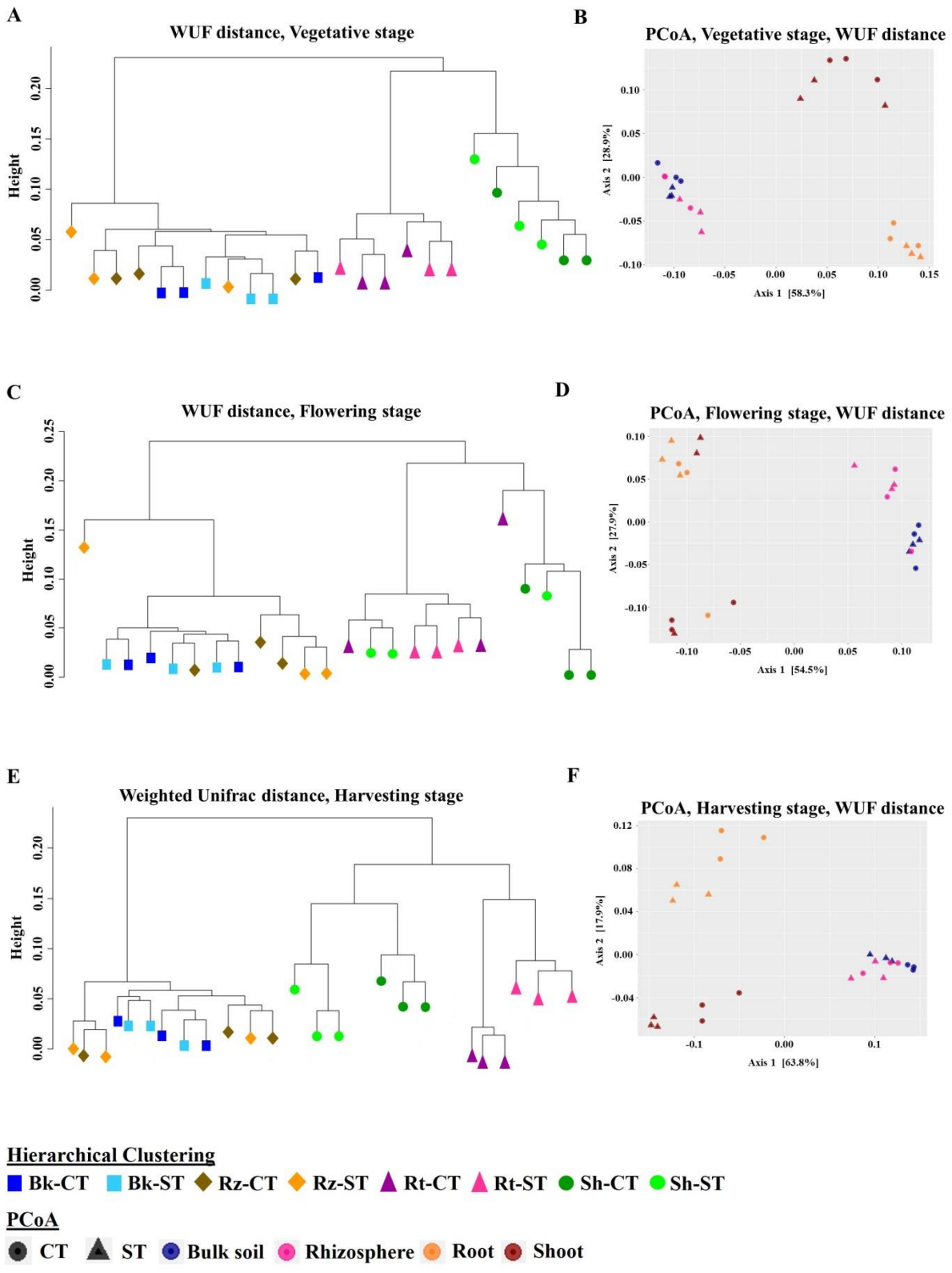


Figure A5.5: Beta diversity analysis of the bacterial communities associated with bulk soil, rhizosphere, root and shoot microhabitats under CT and ST tillage practices at different growth stages. Hierarchical clustering (A, C & E at vegetative, flowering and harvesting

stage respectively) and PCoA (B, D & F at vegetative, flowering and harvesting stage respectively) based on WUF distance calculated using counts per million transformed OTU abundances. In hierarchical graphs shapes presented the different compartments and associated dark colours indicate the CT samples and light colour indicate the ST sample of respective compartment. Whereas, in PCoA graphs, colours define the compartments, while shapes depict the indicated tillage practices. Statistical results of beta diversity are displayed in Excel sheet_WS-5.

Appendix A6

Table A6.1 Crop rotation history under conventional and conservation tillage practices

Year	Conventional tillage (CT)					Conservation strip tillage (ST)				
2012-13	Wheat	Barley	Oats	WOSR	Wheat	Wheat	Barley	Oats	WOSR	Wheat
2013-14	Barley	WOSR	Wheat	Wheat	Wheat	Barley	WOSR	Wheat	Wheat	Wheat
2014-15	**WOSR**	**Wheat**	Barley	Oats	**Wheat**	**WOSR**	**Wheat**	Barley	Oats	**Wheat**
2015-16	**Wheat**	Oats	**WOSR**	Wheat	**Wheat**	**Wheat**	Oats	**WOSR**	Wheat	**Wheat**

*Samples collected in year 2014-15 and 2015-16 were highted with colour. The text highlited with bold letters describe the WOSR and wheat samples collected for analysis in each year and show the previous crop history.

Table A6.2 Crop management practices used for the wheat and WOSR winter crops

Crops Name	Variety	Sowing date	Harvest date	Seed rate Seeds m^{-2}	Fertilizer application kg/ha			Fungicide application	Herbicide application	Insecticide application
					N	P	K			
WOSR	Compass	2/9/14	27/7/15	Year *14/15* 50	225	25.2	108	Proline 0.4 L ha^{-1} (in year 2014/15) & 0.6 L ha^{-1} (in year 2015/16) for Light leaf spot, Filan 0.5 kg ha^{-1} for Phoma stem canker	Katamaran 2L ha^{-1} (Pre-sowing), Falcon 1L ha^{-1} (Post-sowing)	Slug pellets 5kg ha^{-1} (in year 2014/15) & 4 kg ha^{-1} (in year 2015/16) Sumi Alpha for Flea Beetle
		9/9/15	25/7/16	Year *15/16* 60						
Wheat (R) Wheat (M)	J B Diego	30/9/14	25/8/15	300	225	25.2	108	Caldrum + Bravo (2.5 +1 L/ha), Adexar + Bravo (1.6 + 1 L ha^{-1}), Prosaro 1 L ha^{-1} for Septoria disease	Roundup 0.4 L ha^{-1} (Pre-sowing), Alister 1 L ha^{-1} (Post-sowing), Pacifica 0.5 kg ha^{-1}	Sumi Alpha for Aphids
		30/9/15	27/8/16							

*(R) means wheat in rotation where the previous crop was WOSR and (M) means continuously growing wheat or wheat monoculture where previous crop was wheat.

Table A6.3 Physical and chemical characterisation of the soil substrates

Mineral content ($g \cdot kg^{-1}$)	Rotation CT	Rotation ST	Monoculture CT	Monoculture ST
Total carbon	3.83	3.87	3.72	3.75
Total nitrogen	0.27	0.27	0.26	0.26
Soil organic matter	6.70	6.82	6.67	6.69
Soil organic carbon	2.74	2.77	2.71	2.71
C/N ratio	12.87	13.12	12.45	12.46
pH	6.52	7.12	6.80	7.05
Soil classification	Sandy clay loam			

*Rotation = WOSR and wheat crops were grown in rotation. Monoculture = wheat was continuosly grown in the same plot under CT and ST practices.

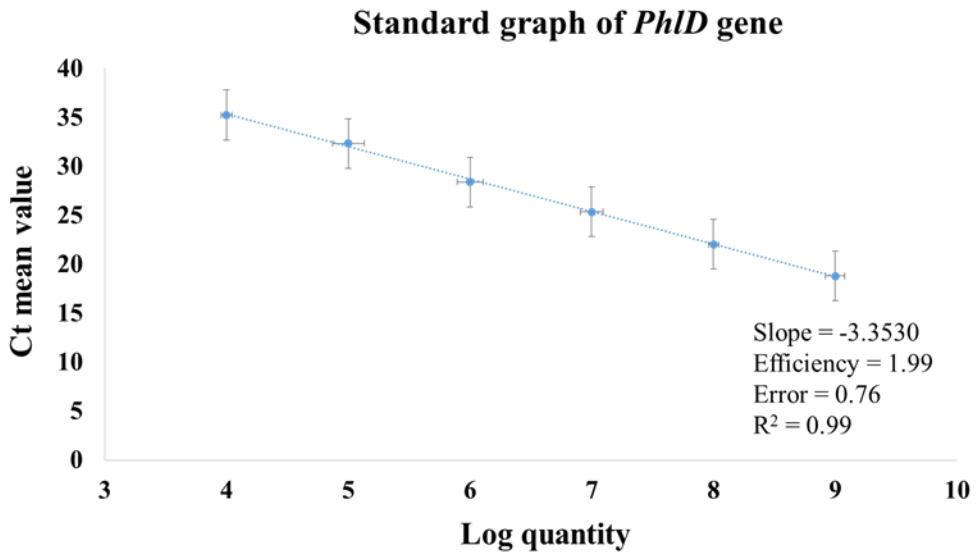
Table A6.4 Climate and weather conditions during the experiment

Months	Max air temp ºC		Min air temp °C		Mean air temp °C	Total rainfall (mm)	Mean wind Speed (ms^{-1})	Max wind speed (ms^{-1})	Solar radiation (Wm^{-2})	Humidity (%)	CBL pressure (Pa)	Soil temperature °C at			
	High	Low	Low	High								5cm	10cm	20cm	30cm
2014/15															
September	22.8	15.6	4.4	14	14.3	0.61	2.18	07.6	1146	82.05	1013	16.1	16.1	15.9	15.6
October	17.7	13.2	0.5	14.6	11.4	4.46	4.41	12.5	0613	85.14	1001	11.4	11.6	11.9	11.9
November	14.2	4.2	-2.1	8.1	7.2	5.52	3.02	09.5	0300	90.49	0996	7.2	7.5	8.1	8.3
December	13.5	3.0	-5.3	10.8	5.6	1.54	4.32	11.7	0230	87.94	1011	4.5	4.8	5.4	5.5
January	16.2	2.1	-4.8	8.5	4.99	2.13	5.33	14.3	0285	85.05	1004	4.0	4.1	4.7	4.8
February	12.8	3.3	-5.6	7.2	4.30	1.30	3.99	11.7	0480	86.54	1009	4.0	4.1	4.3	4.3
March	14.2	5.6	-2.2	9.2	6.24	1.73	4.76	13.5	1005	80.05	1012	6.5	6.5	6.5	6.4
April	18.9	9.6	-0.4	7.2	8.65	0.88	3.48	10.4	1638	77.59	1014	10.9	10.9	10.5	10.1
May	19.2	9.6	1.6	11.2	10.21	2.88	4.54	12.5	1629	79.04	1006	12.6	12.5	12.1	11.8
June	24.0	11.9	3.6	15.7	13.43	0.99	4.04	10.9	2029	74.85	1012	17.4	17.2	16.4	15.9
July	23.4	16.5	6.4	13.8	14.58	2.56	4.04	11.4	1485	80.19	1005	16.7	16.6	16.5	16.1
August	23.0	14.8	6.0	13.9	14.64	2.68	3.57	10.9	1408	80.02	1005	16.7	16.6	16.4	16.0
2015/16															
September	19.4	13.3	3.8	13.3	12.43	0.92	3.15	09.1	0993	81.53	1010	13.7	13.7	13.8	13.6
October	19.1	11.1	-0.3	12.3	10.21	1.83	2.93	09.3	0523	87.17	1010	10.9	11.0	11.2	11.2
November	17.7	5.6	-2.1	13.9	9.21	3.67	5.05	15.2	0255	86.56	1005	8.8	8.9	9.4	9.4
December	14.1	6.3	-0.1	11.7	8.57	8.74	6.22	17.5	0146	89.45	1001	7.7	7.8	8.1	8.2
January	13.6	4.9	-3.1	11.9	5.91	3.58	4.74	13.5	0230	90.77	0996	5.2	5.3	5.7	5.8
February	13.1	5.1	-3.2	7.8	4.85	3.30	4.49	12.7	0440	87.28	1001	4.8	4.9	5.4	5.4

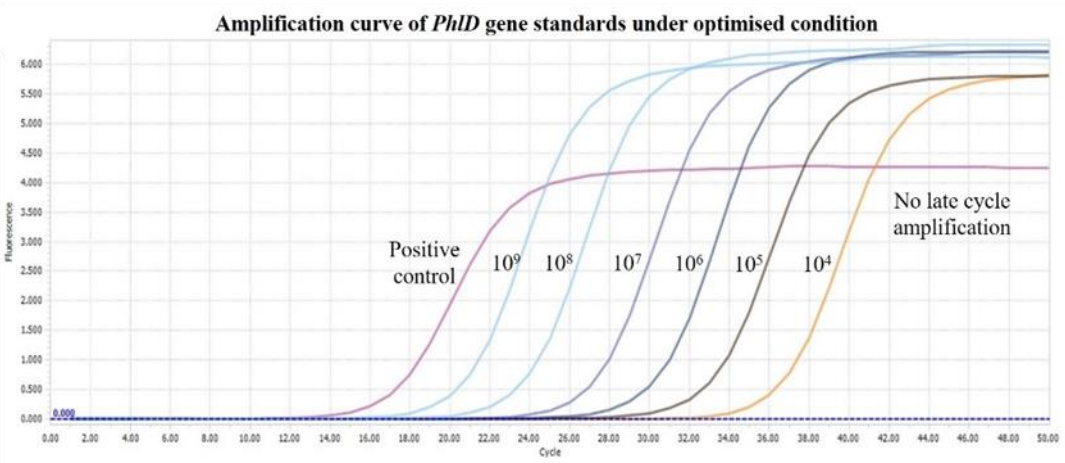
March	14.1	5.5	-3.5	7.4	6.13	1.31	3.77	10.9	0862	83.28	1007	6.5	6.5	6.6	6.5
April	17.2	7.4	-1.4	7.0	7.51	2.14	3.74	11.7	1323	79.48	1005	9.3	9.2	9.0	8.7
May	21.9	11.6	3.4	12.4	12.36	1.99	3.26	9.74	1742.57	80.65	1007	15.3	15.0	14.2	13.7
June	23.7	14.3	4.7	15.1	15.07	2.06	3.21	9.52	1604.07	83.67	1007	18.1	17.8	17.4	17.0
July	27.5	15.3	6.8	15.4	15.99	0.95	3.63	10.41	1515.66	83.31	1008	18.7	18.5	18.0	17.6
August	23.5	15.9	7.8	15.4	16.03	1.48	3.86	10.55	1295.70	82.10	1010	17.9	17.8	17.6	17.3

*Parameters were recorded for example; temperature in degree Celsius, rainfall in millimetres, wind speed in meters per second, humidity in percentage, Convective Boundary Layer (CBL) atmospheric pressure in Pascal (Pa), and solar radiation in Watt per square meter. WOSR and wheat crops were sawn in month of September and harvested in July and August respectively. Maximum and minimum air temperature was reported from the range of high to low.

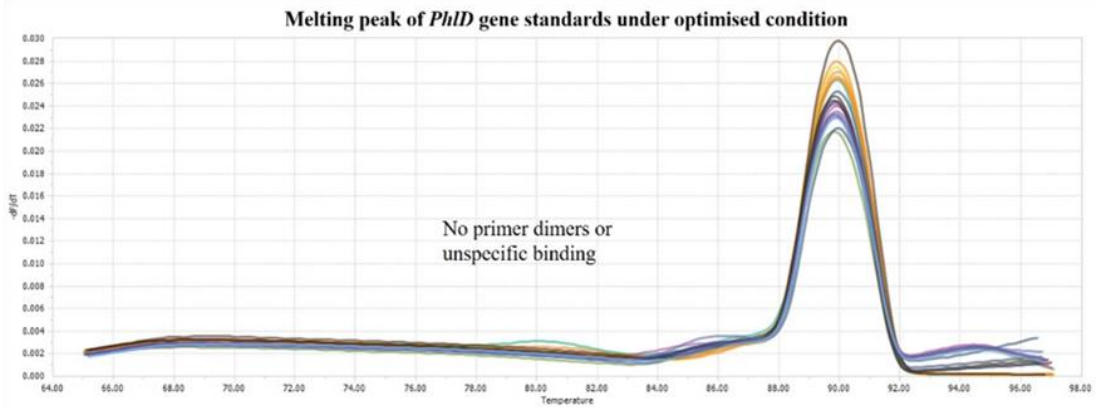
A


Standard graph of *PhlD* gene
Ct mean value
Log quantity
Slope = -3.3530
Efficiency = 1.99
Error = 0.76
R^2 = 0.99

B


Amplification curve of *PhlD* gene standards under optimised condition
Positive control
10^9
10^8
10^7
10^6
10^5
10^4
No late cycle amplification
Fluorescence
Cycle

C


Melting peak of *PhlD* gene standards under optimised condition
No primer dimers or unspecific binding
Temperature

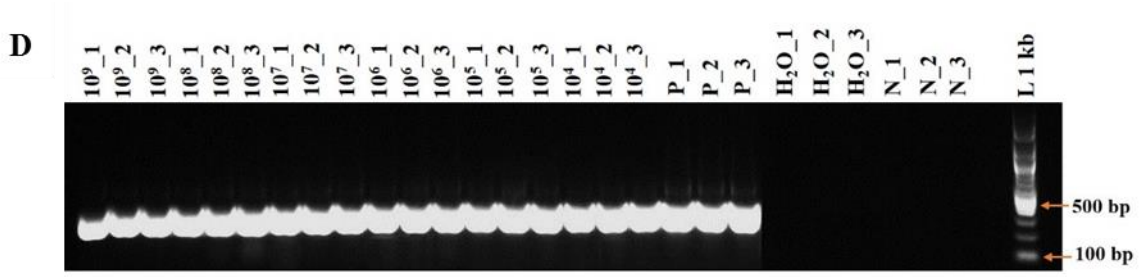


Figure A6.1 Standard curve was generated using 10-fold dilution of the bacterial DNA from *P. fluorescens* strain F113. Each dilution was analysed in triplicate. **A**, Standard curve with the Ct plotted against the log of the starting quantity of DNA for each dilution. **B**, Amplification curves of the dilution series. **C**, Melting pick of each dilution D, PCR amplification on agarose gel of each dilution, positive, negative and water control in triplicate. (**P**) positive control *P. fluorescens* F113 DNA, (**N**) negative control *E.coli* DNA.

Table A6.5 Ct value and associated calculations for *PhlD* gene copy number per gram of rhizosphere soil sample from year 2014/15.

Growth Stage	Crop type	Tillage type	Mean Ct value	Estimated log value	Copy number	Soil in g	DNA con. per µL	Total DNA con. in 50 µL	Total copy no.	Copy no in g soil	Log value g soil^{-1}
Vegetative	WOSR	CT	34.343	4.307	20261	0.25	81.3	4065	8235917	32943668	7.52
Vegetative	WOSR	CT	35.567	3.938	8674	0.25	86.0	4300	3730032	14920128	7.17
Vegetative	WOSR	CT	33.527	4.553	35694	0.25	83.0	4150	14812863	59251450	7.77
Vegetative	WOSR	ST	29.420	5.789	615623	0.25	89.0	4450	273952454	1095809816	9.04
Vegetative	WOSR	ST	27.783	6.282	1915129	0.25	95.0	4750	909686199	3638744796	9.56
Vegetative	WOSR	ST	30.280	5.530	339098	0.25	91.0	4550	154289809	617159236	8.79
Vegetative	Wheat	CT	31.157	5.266	184636	0.25	76.7	3835	70808061	283232243	8.45
Vegetative	Wheat	CT	31.403	5.192	155609	0.25	84.3	4215	65589021	262356083	8.42
Vegetative	Wheat	CT	31.733	5.093	123781	0.25	80.8	4040	50007518	200030073	8.30
Vegetative	Wheat	ST	26.757	6.591	3902825	0.25	89.7	4485	1750416971	7001667885	9.85
Vegetative	Wheat	ST	30.373	5.502	317847	0.25	92.3	4615	146686511	586746046	8.77
Vegetative	Wheat	ST	28.680	6.012	1028410	0.25	90.4	4520	464841518	1859366072	9.27
Vegetative	Wheat (C)	CT	34.713	4.195	15676	0.25	95.8	4790	7508640	30034560	7.48
Vegetative	Wheat (C)	CT	33.293	4.623	41962	0.25	88.7	4435	18610282	74441127	7.87
Vegetative	Wheat (C)	CT	32.527	4.854	71407	0.25	86.2	4310	30776453	123105811	8.09
Vegetative	Wheat (C)	ST	30.493	5.466	292469	0.25	96.7	4835	141408953	565635811	8.75
Vegetative	Wheat (C)	ST	30.650	5.419	262362	0.25	96.0	4800	125933547	503734188	8.70
Vegetative	Wheat (C)	ST	29.537	5.754	567781	0.25	91.5	4575	259759877	1039039507	9.02
Flowering	WOSR	CT	37.547	3.342	2198	0.25	72.4	3620	795564	3182254	6.50
Flowering	WOSR	CT	36.507	3.655	4520	0.25	77.3	3865	1747078	6988311	6.84
Flowering	WOSR	CT	37.623	3.319	2084	0.25	74.0	3700	771045	3084181	6.49

Flowering	WOSR	ST	33.587	4.535	34239	0.25	100	5000	17119532	68478126	7.84
Flowering	WOSR	ST	34.360	4.302	20028	0.25	94.6	4730	9473127	37892509	7.58
Flowering	WOSR	ST	32.143	4.969	93150	0.25	97.0	4850	45177689	180710757	8.26
Flowering	Wheat	CT	36.477	3.664	4615	0.25	78.1	3905	1802263	7209054	6.86
Flowering	Wheat	CT	35.323	4.012	10269	0.25	84.9	4245	4359149	17436596	7.24
Flowering	Wheat	CT	35.850	3.853	7127	0.25	87.3	4365	3111017	12444068	7.09
Flowering	Wheat	ST	34.490	4.262	18301	0.25	85.7	4285	7842120	31368482	7.50
Flowering	Wheat	ST	31.697	5.104	126969	0.25	83.9	4195	53263302	213053207	8.33
Flowering	Wheat	ST	36.407	3.685	4845	0.25	91.3	4565	2211661	8846644	6.95
Flowering	Wheat (C)	CT	37.423	3.379	2394	0.25	79.2	3960	947989	3791957	6.58
Flowering	Wheat (C)	CT	35.683	3.903	8000	0.25	81.7	4085	3268150	13072599	7.12
Flowering	Wheat (C)	CT	37.367	3.396	2490	0.25	70.9	3545	882652	3530609	6.55
Flowering	Wheat (C)	ST	31.367	5.203	159616	0.25	99.9	4995	79728068	318912273	8.50
Flowering	Wheat (C)	ST	35.787	3.872	7447	0.25	93.7	4685	3488997	13955988	7.14
Flowering	Wheat (C)	ST	33.587	4.535	34239	0.25	87.6	4380	14996710	59986839	7.78
Harvesting	WOSR	CT	36.507	3.655	4520	0.25	89.3	4465	2018293	8073171	6.91
Harvesting	WOSR	CT	39.510	2.751	563	0.25	73.0	3650	205589	822357	5.92
Harvesting	WOSR	CT	32.730	4.793	62017	0.25	84.0	4200	26046938	104187750	8.02
Harvesting	WOSR	ST	28.453	6.080	1203448	0.25	82.1	4105	494015293	1976061172	9.30
Harvesting	WOSR	ST	27.523	6.360	2293491	0.25	88.0	4400	1009136176	4036544705	9.61
Harvesting	WOSR	ST	26.690	6.611	4087481	0.25	78.0	3900	1594117642	6376470567	9.80
Harvesting	Wheat	CT	0.000	0.000	0	0.25	70.2	3510	0	0	0.00
Harvesting	Wheat	CT	0.000	0.000	0	0.25	75.9	3795	0	0	0.00
Harvesting	Wheat	CT	0.000	0.000	0	0.25	77.7	3885	0	0	0.00
Harvesting	Wheat	ST	35.690	3.901	7963	0.25	81.1	4055	3229186	12916744	7.11
Harvesting	Wheat	ST	37.157	3.459	2880	0.25	80.0	4000	1152060	4608238	6.66

Harvesting	Wheat	ST	38.847	2.950	892	0.25	85.5	4275	381423	1525692	6.18
Harvesting	Wheat (C)	CT	39.810	2.660	457	0.25	88.3	4415	201973	807892	5.91
Harvesting	Wheat (C)	CT	40.307	2.511	324	0.25	80.6	4030	130646	522584	5.72
Harvesting	Wheat (C)	CT	41.420	2.176	150	0.25	86.0	4300	64414	257655	5.41
Harvesting	Wheat (C)	ST	36.440	3.675	4734	0.25	81.0	4050	1917319	7669277	6.88
Harvesting	Wheat (C)	ST	37.260	3.428	2681	0.25	89.0	4450	1193043	4772172	6.68
Harvesting	Wheat (C)	ST	39.293	2.816	655	0.25	86.0	4300	281466	1125863	6.05

Table A6.6 Ct value and associated calculations for *PhlD* gene copy number per gram of rhizosphere soil sample from year 2015/16.

Growth Stage	Crop type	Tillage type	Mean Ct value	Estimated log value	Copy number	Soil in g	DNA con. per µL	Total DNA con. in 50 µL	Total copy no.	Copy no in g soil	Log value g soil^{-1}
Vegetative	CT	WOSR	33.287	4.625	42157	0.25	86.0	4300	18127397	72509588	7.86
Vegetative	CT	WOSR	35.687	3.902	7982	0.25	80.6	4030	3216704	12866816	7.11
Vegetative	CT	WOSR	35.377	3.995	9896	0.25	88.3	4415	4369114	17476456	7.24
Vegetative	ST	WOSR	29.533	5.755	569095	0.25	86.0	4300	244710870	978843481	8.99
Vegetative	ST	WOSR	27.693	6.309	2038457	0.25	89.0	4450	907113317	3628453269	9.56
Vegetative	ST	WOSR	30.437	5.483	304190	0.25	81.0	4050	123197144	492788575	8.69
Vegetative	CT	Wheat	34.400	4.290	19480	0.25	85.5	4275	8327648	33310590	7.52
Vegetative	CT	Wheat	33.600	4.531	33924	0.25	80.0	4000	13569584	54278335	7.73
Vegetative	CT	Wheat	33.437	4.580	37992	0.25	81.8	4090	15538809	62155238	7.79
Vegetative	ST	Wheat	27.517	6.363	2304118	0.25	77.7	3885	895149949	3580599797	9.55
Vegetative	ST	Wheat	31.663	5.114	129937	0.25	75.9	3795	49311277	197245106	8.30
Vegetative	ST	Wheat	28.307	6.125	1332281	0.25	70.2	3510	467630763	1870523050	9.27
Vegetative	CT	Wheat (C)	35.467	3.968	9297	0.25	78.0	3900	3625966	14503864	7.16
Vegetative	CT	Wheat (C)	35.577	3.935	8615	0.25	88.0	4400	3790402	15161608	7.18
Vegetative	CT	Wheat (C)	34.330	4.311	20449	0.25	82.1	4105	8394212	33576846	7.53
Vegetative	ST	Wheat (C)	30.090	5.588	386852	0.25	84.0	4200	162477680	649910721	8.81
Vegetative	ST	Wheat (C)	29.427	5.787	612784	0.25	73.0	3650	223666210	894664841	8.95
Vegetative	ST	Wheat (C)	30.427	5.486	306307	0.25	89.3	4465	136766138	547064552	8.74
Flowering	CT	WOSR	41.177	2.249	177	0.25	87.6	4380	77672	310688	5.49
Flowering	CT	WOSR	40.273	2.521	332	0.25	93.7	4685	155432	621726	5.79
Flowering	CT	WOSR	39.450	2.769	587	0.25	99.9	4995	293300	1173200	6.07

Flowering	ST	WOSR	36.633	3.617	4140	0.25	70.9	3545	1467686	5870745	6.77
Flowering	ST	WOSR	39.470	2.763	579	0.25	81.7	4085	236562	946250	5.98
Flowering	ST	WOSR	37.617	3.321	2094	0.25	79.2	3960	829050	3316202	6.52
Flowering	CT	Wheat	47.467	0.355	2	0.25	90.4	4520	1023	4090	3.61
Flowering	CT	Wheat	45.480	0.953	9	0.25	92.3	4615	4140	16560	4.22
Flowering	CT	Wheat	48.553	0.027	1	0.25	89.7	4485	478	1910	3.28
Flowering	ST	Wheat	33.360	4.603	40067	0.25	87.3	4365	17489077	69956306	7.84
Flowering	ST	Wheat	31.500	5.163	145520	0.25	84.9	4245	61773194	247092776	8.39
Flowering	ST	Wheat	35.150	4.064	11580	0.25	78.1	3905	4522145	18088579	7.26
Flowering	CT	Wheat (C)	45.500	0.947	9	0.25	90.4	4520	3999	15996	4.20
Flowering	CT	Wheat (C)	48.200	0.134	1	0.25	69.4	3468	472	1887	3.28
Flowering	CT	Wheat (C)	48.630	0.004	1	0.25	70.1	3506	354	1416	3.15
Flowering	ST	Wheat (C)	40.450	2.468	294	0.25	74.0	3700	108600	434398	5.64
Flowering	ST	Wheat (C)	47.730	0.275	2	0.25	77.3	3865	728	2914	3.46
Flowering	ST	Wheat (C)	48.497	0.044	1	0.25	72.4	3620	401	1604	3.21
Harvesting	CT	WOSR	31.690	5.106	127557	0.25	91.5	4575	58357259	233429037	8.37
Harvesting	CT	WOSR	33.530	4.552	35611	0.25	96.0	4800	17093394	68373575	7.83
Harvesting	CT	WOSR	33.533	4.551	35529	0.25	96.7	4835	17178281	68713125	7.84
Harvesting	ST	WOSR	27.713	6.303	2010382	0.25	86.2	4310	866474516	3465898064	9.54
Harvesting	ST	WOSR	26.690	6.611	4087481	0.25	88.7	4435	1812797882	7251191529	9.86
Harvesting	ST	WOSR	27.617	6.332	2149759	0.25	95.8	4790	1029734449	4118937798	9.61
Harvesting	CT	Wheat	47.467	0.355	2	0.25	90.4	4520	1023	4090	3.61
Harvesting	CT	Wheat	45.480	0.953	9	0.25	92.3	4615	4140	16560	4.22
Harvesting	CT	Wheat	48.553	0.027	1	0.25	89.7	4485	478	1910	3.28
Harvesting	ST	Wheat	39.703	2.692	493	0.25	80.8	4040	199006	796025	5.90
Harvesting	ST	Wheat	37.533	3.346	2218	0.25	84.3	4215	934930	3739721	6.57

Harvesting	ST	Wheat	31.770	5.082	120673	0.25	76.7	3835	46278271	185113084	8.27
Harvesting	CT	Wheat (C)	47.293	0.407	3	0.25	91.0	4550	1161	4643	3.67
Harvesting	CT	Wheat (C)	47.487	0.349	2	0.25	95.0	4750	1060	4239	3.63
Harvesting	CT	Wheat (C)	44.873	1.136	14	0.25	89.0	4450	6080	24320	4.39
Harvesting	ST	Wheat (C)	35.303	4.018	10412	0.25	83.0	4150	4321108	17284432	7.24
Harvesting	ST	Wheat (C)	36.617	3.622	4188	0.25	86.0	4300	1800962	7203849	6.86
Harvesting	ST	Wheat (C)	37.593	3.328	2128	0.25	81.3	4065	864914	3459658	6.54

Table A6.7 Ct value and associated calculations for *PhlD* gene copy number per gram of root sample from year 2014/15.

Growth Stage	Crop type	Tillage type	Mean Ct value	Estimated log value	Copy number	Root wt. in g	DNA con. per µL	Total DNA con. in 50 µL	Total copy number	Copy number in g root	Log value g root^{-1}
Vegetative	WOSR	CT	33.513	4.557	36025	0.5	118	5900	21254860	42509720	7.63
Vegetative	WOSR	CT	30.633	5.424	265411	0.5	108	5400	143322090	286644179	8.46
Vegetative	WOSR	CT	30.923	5.337	217063	0.5	112	5600	121555268	243110536	8.39
Vegetative	WOSR	ST	27.660	6.319	2086123	0.5	102	5100	1063922649	2127845298	9.33
Vegetative	WOSR	ST	27.840	6.265	1841335	0.5	100	5000	920667498	1841334996	9.27
Vegetative	WOSR	ST	27.613	6.333	2154783	0.5	110	5500	1185130817	2370261634	9.37
Vegetative	Wheat	CT	35.833	3.858	7210	0.5	146	7300	5263325	10526650	7.02
Vegetative	Wheat	CT	35.260	4.031	10730	0.5	151	7550	8101097	16202194	7.21
Vegetative	Wheat	CT	37.453	3.370	2345	0.5	150	7500	1758470	3516940	6.55
Vegetative	Wheat	ST	31.617	5.128	134211	0.5	130	6500	87237158	174474315	8.24
Vegetative	Wheat	ST	33.733	4.490	30928	0.5	136	6800	21031104	42062207	7.62
Vegetative	Wheat	ST	32.450	4.877	75306	0.5	121	6050	45560120	91120241	7.96
Vegetative	Wheat (C)	CT	33.690	4.503	31872	0.5	107	5350	17051272	34102544	7.53
Vegetative	Wheat (C)	CT	32.223	4.945	88123	0.5	111	5550	48908374	97816749	7.99
Vegetative	Wheat (C)	CT	35.437	3.977	9493	0.5	114	5700	5410887	10821775	7.03
Vegetative	Wheat (C)	ST	31.657	5.116	130540	0.5	105	5250	68533267	137066535	8.14
Vegetative	Wheat (C)	ST	34.520	4.253	17925	0.5	100	5000	8962273	17924546	7.25

Vegetative	Wheat (C)	ST	36.590	3.630	4266	0.5	103	5150	2197223	4394445	6.64
Flowering	WOSR	CT	37.447	3.372	2355	0.5	145	7250	1707731	3415461	6.53
Flowering	WOSR	CT	36.203	3.747	5578	0.5	142	7100	3960682	7921365	6.90
Flowering	WOSR	CT	35.353	4.002	10057	0.5	140	7000	7040241	14080483	7.15
Flowering	WOSR	ST	35.547	3.944	8796	0.5	120	6000	5277380	10554760	7.02
Flowering	WOSR	ST	34.203	4.349	22326	0.5	113	5650	12614235	25228470	7.40
Flowering	WOSR	ST	33.450	4.576	37643	0.5	109	5450	20515192	41030385	7.61
Flowering	Wheat	CT	35.763	3.879	7569	0.5	112	5600	4238438	8476877	6.93
Flowering	Wheat	CT	35.563	3.939	8695	0.5	119	5950	5173267	10346534	7.01
Flowering	Wheat	CT	35.530	3.949	8898	0.5	102	5100	4537916	9075832	6.96
Flowering	Wheat	ST	36.217	3.742	5527	0.5	135	6750	3730784	7461568	6.87
Flowering	Wheat	ST	35.357	4.001	10034	0.5	129	6450	6472103	12944205	7.11
Flowering	Wheat	ST	38.657	3.008	1018	0.5	137	6850	697236	1394472	6.14
Flowering	Wheat (C)	CT	35.580	3.934	8595	0.5	152	7600	6531942	13063885	7.12
Flowering	Wheat (C)	CT	37.323	3.409	2566	0.5	146	7300	1873036	3746072	6.57
Flowering	Wheat (C)	CT	36.657	3.610	4074	0.5	139	6950	2831229	5662458	6.75
Flowering	Wheat (C)	ST	33.543	4.548	35284	0.5	116	5800	20464433	40928866	7.61
Flowering	Wheat (C)	ST	32.617	4.827	67087	0.5	122	6100	40923012	81846024	7.91
Flowering	Wheat (C)	ST	34.307	4.318	20782	0.5	110	5500	11430264	22860529	7.36
Harvesting	WOSR	CT	34.427	4.282	19123	0.5	120	6000	11473787	22947574	7.36
Harvesting	WOSR	CT	35.287	4.023	10533	0.5	107	5350	5635338	11270676	7.05

Harvesting	WOSR	CT	36.520	3.651	4479	0.5	116	5800	2597619	5195237	6.72
Harvesting	WOSR	ST	27.633	6.327	2125057	0.5	142	7100	1508790373	3017580745	9.48
Harvesting	WOSR	ST	27.797	6.278	1897504	0.5	139	6950	1318765151	2637530303	9.42
Harvesting	WOSR	ST	24.823	7.174	14914310	0.5	136	6800	10141730759	20283461518	10.31
Harvesting	Wheat	CT	34.937	4.128	13427	0.5	150	7500	10070015	20140029	7.30
Harvesting	Wheat	CT	36.333	3.707	5098	0.5	155	7750	3950607	7901214	6.90
Harvesting	Wheat	CT	37.650	3.311	2046	0.5	144	7200	1472923	2945845	6.47
Harvesting	Wheat	ST	36.010	3.805	6379	0.5	159	7950	5071089	10142178	7.01
Harvesting	Wheat	ST	36.447	3.673	4712	0.5	153	7650	3604900	7209799	6.86
Harvesting	Wheat	ST	33.343	4.608	40532	0.5	151	7550	30601926	61203851	7.79
Harvesting	Wheat (C)	CT	36.663	3.608	4055	0.5	134	6700	2716798	5433595	6.74
Harvesting	Wheat (C)	CT	38.300	3.115	1303	0.5	133	6650	866805	1733610	6.24
Harvesting	Wheat (C)	CT	35.440	3.976	9471	0.5	138	6900	6534899	13069799	7.12
Harvesting	Wheat (C)	ST	30.630	5.425	266025	0.5	106	5300	140993495	281986990	8.45
Harvesting	Wheat (C)	ST	28.727	5.998	995664	0.5	111	5550	552593502	1105187005	9.04
Harvesting	Wheat (C)	ST	28.963	5.927	844969	0.5	105	5250	443608475	887216949	8.95

Table A6.8 Ct value and associated calculations for *PhlD* gene copy number per gram of root sample from year 2015/16.

Growth Stage	Crop type	Tillage type	Mean Ct value	Estimated log value	Copy number	Root wt. in g	DNA con. per µL	Total DNA con. in 50 µL	Total copy number	Copy number in g root	Log value g root^{-1}
Vegetative	WOSR	CT	33.643	4.517	32919.76	0.5	142	7100	23373031	46746062	7.67
Vegetative	WOSR	CT	32.427	4.884	76534.34	0.5	140	7000	53574037	107148073	8.03
Vegetative	WOSR	CT	31.290	5.226	168330.9	0.5	136	6800	114465039	228930077	8.36
Vegetative	WOSR	ST	29.903	5.644	440310.6	0.5	130	6500	286201903	572403805	8.76
Vegetative	WOSR	ST	33.510	4.558	36108.55	0.5	135	6750	24373272	48746545	7.69
Vegetative	WOSR	ST	33.473	4.569	37038.4	0.5	121	6050	22408234	44816468	7.65
Vegetative	Wheat	CT	35.587	3.932	8555.021	0.5	147	7350	6287941	12575881	7.10
Vegetative	Wheat	CT	34.597	4.230	16996.52	0.5	152	7600	12917354	25834708	7.41
Vegetative	Wheat	CT	36.670	3.606	4036.22	0.5	144	7200	2906078	5812157	6.76
Vegetative	Wheat	ST	32.433	4.882	76181.35	0.5	157	7850	59802359	119604719	8.08
Vegetative	Wheat	ST	31.620	5.127	133901.2	0.5	145	7250	97078336	194156672	8.29
Vegetative	Wheat	ST	33.730	4.491	30999.66	0.5	152	7600	23559744	47119489	7.67
Vegetative	Wheat (C)	CT	33.756	4.484	30445.78	0.5	111	5550	16897407	33794813	7.53
Vegetative	Wheat (C)	CT	34.555	4.243	17494.76	0.5	120	6000	10496854	20993708	7.32
Vegetative	Wheat (C)	CT	32.964	4.722	52727.62	0.5	109	5450	28736554	57473108	7.76
Vegetative	Wheat (C)	ST	34.630	4.220	16608.16	0.5	118	5900	9798817	19597634	7.29
Vegetative	Wheat (C)	ST	33.437	4.580	37992.2	0.5	126	6300	23935085	47870171	7.68
Vegetative	Wheat (C)	ST	30.320	5.518	329822.2	0.5	122	6100	201191516	402383033	8.60
Flowering	WOSR	CT	36.643	3.614	4111.549	0.5	146	7300	3001431	6002862	6.78
Flowering	WOSR	CT	34.703	4.198	15784.73	0.5	153	7650	12075321	24150643	7.38
Flowering	WOSR	CT	34.780	4.175	14967.49	0.5	150	7500	11225620	22451240	7.35

Flowering	WOSR	ST	34.510	4.256	18049.27	0.5	143	7150	12905229	25810458	7.41
Flowering	WOSR	ST	35.440	3.976	9470.869	0.5	136	6800	6440191	12880381	7.11
Flowering	WOSR	ST	31.443	5.180	151351.8	0.5	140	7000	105946251	211892502	8.33
Flowering	Wheat	CT	35.470	3.967	9275.884	0.5	129	6450	5982945	11965890	7.08
Flowering	Wheat	CT	35.600	3.928	8476.289	0.5	133	6650	5636732	11273464	7.05
Flowering	Wheat	CT	35.493	3.960	9127.008	0.5	125	6250	5704380	11408761	7.06
Flowering	Wheat	ST	32.563	4.843	69614.4	0.5	157	7850	54647307	109294615	8.04
Flowering	Wheat	ST	34.697	4.200	15857.87	0.5	153	7650	12131273	24262546	7.38
Flowering	Wheat	ST	35.623	3.921	8340.247	0.5	160	8000	6672197	13344395	7.13
Flowering	Wheat (C)	CT	32.807	4.769	58805.67	0.5	100	5000	29402834	58805667	7.77
Flowering	Wheat (C)	CT	35.480	3.964	9211.785	0.5	141	7050	6494308	12988617	7.11
Flowering	Wheat (C)	CT	33.737	4.489	30856.69	0.5	111	5550	17125462	34250924	7.53
Flowering	Wheat (C)	ST	33.023	4.704	50613.96	0.5	145	7250	36695121	73390241	7.87
Flowering	Wheat (C)	ST	35.443	3.975	9449.003	0.5	139	6950	6567057	13134114	7.12
Flowering	Wheat (C)	ST	33.890	4.443	27744.25	0.5	142	7100	19698414	39396829	7.60
Harvesting	WOSR	CT	35.303	4.018	10412.31	0.5	143	7150	7444801	14889601	7.17
Harvesting	WOSR	CT	36.550	3.642	4386.447	0.5	138	6900	3026648	6053297	6.78
Harvesting	WOSR	CT	34.493	4.261	18259.08	0.5	136	6800	12416173	24832346	7.40
Harvesting	WOSR	ST	27.520	6.362	2298799	0.5	133	6650	1528701103	3057402206	9.49
Harvesting	WOSR	ST	32.400	4.892	77962.72	0.5	130	6500	50675771	101351542	8.01
Harvesting	WOSR	ST	25.767	6.890	7753860	0.5	128	6400	4962470437	9924940875	10.00
Harvesting	Wheat	CT	34.403	4.289	19434.9	0.5	120	6000	11660942	23321884	7.37
Harvesting	Wheat	CT	35.773	3.876	7516.339	0.5	116	5800	4359476	8718953	6.94
Harvesting	Wheat	CT	36.232	3.738	5468.632	0.5	118	5900	3226493	6452986	6.81
Harvesting	Wheat	ST	35.370	3.997	9941.922	0.5	159	7950	7903828	15807655	7.20
Harvesting	Wheat	ST	36.490	3.660	4572.797	0.5	155	7750	3543917	7087835	6.85

Harvesting	Wheat	ST	31.373	5.201	158879.6	0.5	157	7850	124720469	249440939	8.40
Harvesting	Wheat (C)	CT	29.903	5.644	440310.6	0.5	151	7550	332434518	664869035	8.82
Harvesting	Wheat (C)	CT	30.870	5.353	225240.8	0.5	143	7150	161047191	322094382	8.51
Harvesting	Wheat (C)	CT	31.497	5.164	145856.6	0.5	149	7450	108663195	217326390	8.34
Harvesting	Wheat (C)	ST	29.143	5.873	745819	0.5	132	6600	492240559	984481118	8.99
Harvesting	Wheat (C)	ST	35.343	4.006	10127.47	0.5	128	6400	6481582	12963163	7.11
Harvesting	Wheat (C)	ST	27.817	6.272	1871370	0.5	123	6150	1150892515	2301785030	9.36