EXPLORING BACTERIAL COMMUNITIES AND THEIR FUNCTION FOR SOIL HEALTH UNDER DIFFERENT CROPPING SYSTEMS

BY

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Submitted to the Faculty of Graduate Studies of

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In Partial Fulfillment of the Requirements

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LIST of ABBREVIATIONS

WR: Wheat Rotation
WM: Wheat Monoculture
CR: Canola Rotation
CM: Canola Monoculture
ZT: Zero Tillage
CT: Conventional Tillage
FO: Forage-grain Organic
FC: Forage-grain Conventional
GO: Grain-only Organic
GC: Grain-only Conventional
HPLC: High-Performance Liquid Chromatography
TRFLP: Terminal Restriction Fragment Length Polymorphism
UPGMA: Unweighted Pair Group Method with Arithmetic Mean
PGPR: Plant Growth Promoting Rhizobacteria

GENERAL ABSTRACT

Rhizosphere and soil bacteria are important drivers in nearly all biochemical cycles in terrestrial ecosystems and participate in maintaining health and productivity of soil in agriculturally managed systems. However, the effect of agricultural management systems on bacterial communities is still poorly understood. In this study, cultural methods and advanced molecular methods (terminal restriction fragment length polymorphism (TRFLP) and 454- pyrosequencing) were used to identify shifts in soil and rhizosphere bacterial diversity, community composition, and functions under different cropping systems in Manitoba, Canada. This included monoculture vs. rotation, zero tillage vs. conventional tillage, and organic farming vs. conventional farming.

Results showed that: (1) different cropping systems did not significantly influence the diversity of bacterial communities. However, a significant variation in relative abundances of bacterial communities at both the phylum and genus level was observed among different cropping systems. Compared to conventional farming systems, organic farming system had a higher percentage of the phylum Proteobacteria (many Plant Growth Promoting Rhizobacteria) and a lower percentage of the phylum Actinobacteria. When canola monoculture was compared to wheat-oat-canola-pea rotation, a significantly higher percentage of Proteobacteria and a lower percentage of Actinobacteria were found in the rotational system. Wheat monoculture shared similar bacterial communities with wheat-oat-canola-pea rotation. Zero tillage did not change bacterial community profiles except for an increase in Firmicutes (many PGPR), compared to conventional tillage. At the genus level, significant differences were found for the dominant genera *Pseudomonas*, *Rhizobium*,

Stenotrophomonas, *Brevundimonas*, *Burkholderia*, *Marmoricola*, *Microlunatus*, and *Solirubrobacter*. The bacterial distribution was strongly associated with soil pH. (2) The cropping systems also influenced the antibiotic-producing *Pseudomonas* populations determined through PCR-based screening for the detection of genes involved in the biosynthesis of antibiotics. It was found that pyrrolnitrin- and phenazine- producing *Pseudomonas* spp. were more prevalent in the soil under zero tillage and organic farming systems, while 2,4-DAPG and pyoluteorin-producing strains were not found in this study.

This comprehensive study provided fundamental information on how different cropping systems affect soil and rhizosphere bacterial communities, which can be used to guide Manitoba farmers to choose proper farming systems to maintain soil health and increase PGPR populations in soil.

FOREWORD

This thesis follows the manuscript style outlined by the Department of Plant Science, University of Manitoba. The thesis is presented as three manuscripts, each containing an abstract, introduction, materials and methods, results and discussion. An abstract and general review of the literature precedes the manuscripts, and a general discussion follows the manuscripts.

1.0 GENERAL INTRODUCTION

Over the last few decades, world agriculture has experienced high increases in crop yield. These increases were achieved through high input of inorganic fertilizers and pesticides, and mechanization driven by fossil fuel. These practices have led to serious environmental problems such as depletion of soil quality and health, ocean and ground water pollution, and emergence of resistant pathogens. It is a big challenge to feed the increasing world population on decreasing farmland areas without damaging the environment. It is well known that rhizosphere and soil microorganisms play an important role in maintaining crop and soil health through versatile mechanisms: nutrient cycling and uptake, suppression of plant pathogens, induction of resistance in plant host, and direct stimulation of plant growth (Kloepper et al 2004; Haas and Défago 2005). As such, maintaining biodiversity of microorganisms in soil is an important component of environment-friendly sustainable agriculture strategies.

Organic farming differs from conventional agriculture in the production process and it relies on techniques such as crop rotation, green manure, and biological pest control to maintain the soil productivity instead of chemical fertilizers and pesticides (Zhengfei 2005). Tillage is a common practice in modern agriculture that involves mechanical manipulation of soil to enhance decomposition of crop residues to prepare seedbeds for planting. It also serves as a method of post-emergence weed control and a management strategy to reduce the incidence of diseases and pests. However, extensive tillage leads to soil erosion and environmental pollution. There are two types of tillage systems: conventional tillage and zero tillage [ZT] (directly seeding on the previous crop stubble). It was found that ZT can reduce soil erosion and increase soil organic matter and microbial biomass compared to conventional tillage (Drijer et al 2000; Kabir 2005). One drawback of ZT is that some soilborne plant diseases can reach damaging levels as pathogens survive

on crop residues left on the soil surface (Guo et al 2005). Crop monoculture and rotation are two opposing agricultural practices. Monoculture is defined as the practice of growing the same crop in the same soil year after year, whereas rotation implies temporal sequences in which different crops are grown on the same land (Las 2003). Many studies comparing monoculture to rotation have been concerned with economic and pathological aspects (Shipton 1977, Zentner et al 1995). Canola is the number one cash crop in Canada, contributing $15.4 billion to the Canadian economy (2011, Canada Canola Council). Wheat is another most important crop in Canada, with annual export revenue of $5.4 billion. Owing to the high price per bushel, canola growers in Canada are attempting to grow it in monoculture.

Some studies have demonstrated that agricultural practices affect the diversity and function of rhizosphere and soil microorganisms (Mäder et al 2002; Esperschutz et al 2007; Sugiyama et al 2010). However, the interactions between agricultural practices and soil microbes are still poorly understood due to the complexity of this "black box" and limitations of analytical methods. The methods that have been used to investigate microbial structure and function include culture-dependent and molecular methods. Culture-dependent method only can assess less than 1% microorganisms dwelling in soil. However, this method provides microbial information on morphlogy and other important traits. Molecular methods are powerful tools to explore microbial structure and composition. TRFLP and Pyrosequencing are high-throughput and have the potential cost effectively to assess microbial communties (Singh et al 2009; Roesch et al 2007).

The objective of this study was to explore bacterial structure and functional changes under different cropping practices and systems using both culture-dependant and

molecular methods, and to gain a better understanding of how to build soil holistic ecology to maintain the health and productivity of soil.

2.0 LITERATURE REVIEW

2.1 The effect of cropping systems on bacterial dynamics

2.1.1 The definition of a cropping system

The definition of cropping system may vary in different literatures, however, in general cropping system refers to growing crops on a given piece of land over a fixed period, and the practices and technologies associated with the crops are produced (Zandstra et al 1981). The cropping systems include several components: plant, environment (soil, water) and management (crop rotation, tillage, pesticides and fertilizers application, fumigation).

2.1.2 The relationship between cropping systems and bacteria

The interactions between a cropping system and bacteria are so complex that a complete understanding of all the relationships involved is unlikely to be achieved. However, the relative consequences of plant, soil and crop management that influence changes of microbes can be evaluated. Generally speaking, soil provides a habitat for bacteria, while plants activate and sustain specific bacterial communities through the selective release of exudates and leachates. In turn, soil bacteria play very important roles in various biogeochemical cycles involving the processing of soil structure formation; organic matter decomposition; toxin removal; and the cycling of carbon, nitrogen, phosphorus, iron, and sulphur. In addition, soil bacteria also influence plant health by suppressing phytopathogens, inducing systemic resistance of plants, and improving plant growth (Molin 1997; Filion et al 1999; O'Donnell et al 2001; Jennifer et al 2004; Garbeva et al 2004).

The effect of plants, soil and crop management (crop rotation, tillage, pesticide and fertilizer application, and irrigation) on bacterial population dynamics will be reviewed in the following sections.

2.1.3 The effect of plants on bacterial communities

There is a hypothesis that plant type is a major determinant of bacterial population since the plant provides nutrients and energy to the microbes. Plant roots secrete a wide variety of compounds to attract bacteria, including sugar, ethylene, amino acids, organic acids, vitamins, and enzymes. Bacteria respond differently to the compounds released by plants, therefore exudates of different plants select different bacteria (Smalla et al 2001; Berg et al 2002, 2006; Garbeva et al 2008). In turn, bacteria also affect the growth and development of plants; promoting the hypothesis that the plant- bacterial partnership successfully co-evolved (Sturz and Christie 2003).

2.1.3.1 The interaction between plants and bacteria

Bacterial communities have been grouped into deleterious, neutral, and beneficial categories, based on the perceived relationship they have with plants (Dommergues 1978). Bacteria, which adversely affected crop growth and development, have been termed deleterious ones (Suslow and Schroth 1982; Schippers et al 1986[a]). By contrast, beneficial bacteria are able to increase crop growth and development in different ways. Such bacteria can fix nitrogen available to plant uptake (Reinhold-Hurek and Hurek 1998; James and Olivares 1997), control phyto-pathogens, enhance availability of minerals (Davison 1988; Marschener and Romheld 1994), and alleviate plant stress in soils high in heavy metals (Burd et al 1998). An neutral bacterial community has no effect on the plant.

2.1.3.2 The influence of plants on the bacteria population

Studies have revealed that many phytopathogenic microbes have coevolved with plants, and show a high degree of host specificity (Raaijmakers et al 2009). In contrast, much less is known about the interactions between neutral, and/or beneficial microbes and plants. Several beneficial plant-microbe interactions have been well studied, such as rhizobia, *Azosprillium* and *Pseudomonas* with their hosts (Long 2001; Germida & Siciliano 2001; Haas & Défago 2005; Bashan and de-Bashan 2010).

The most intensively studied invasive beneficial plant-microbe interaction is between legumes and rhizobia and is highly specific (Caetabi-Abiikes and Gresshoff 1991;Denarie et al 1992; Fisher and Long 1992). It was found that there is variability within a plant species to interact with rhizobia. The Gene-for-Gene hypothesis narrows the symbiotic relationship between legume and rhizobia by stating that the interaction between a bacterial strain and a plant cultivar or genotype is either compatible or incompatible (Lie 1978; Young and Matthew 1982; Cregan et al 1989; Lewis-Henderson and Djordjevic 1991). Rosas and his colleagues (1998) investigated host contributions to nodulation by genetically enhanced rhizobium strains. A large collection of bean germplasm was summed for accessions that were preferentially nodulated by the mutant strain KIM5 (Fix^- mutant of the wild- type), when planted in soil containing rhizobia indigenous to Honduras. Fifty genotypes from 820 diverse bean accessions were identified that had either very low or very high preferential nodulation by KIM5. Most of the cultivars with very high preferential nodulation by KIM5 were from Middle American origin suggesting that the isolated breeding and development of bean cultivars in different regions may have lead to selection for nodulation by specific strains. It was found that preferential nodulation by KIM5 is controlled primarily by two Loci. The genetic mapping of the position of the

quantitative trait loci (QTL) conditioning preferential nodulation variation by KIM5 is currently under way. Interestingly, one of these QTL mapped to the location that has a QTL conditioning resistance to common bacterial blight caused by *Xanthomonas campestris* (Nodari et al 1993). It is interesting to observe the genetic proximity and the relationship between host genes that affect pathogenic and beneficial plant-microbe interactions. However, the practical application of biological nitrogen fixation can also be improved through the breeding of plants and should not just focus on nitrogen fixing bacteria such as rhizobia (Bliss 1991).

Non-pathogenic plant-associated microbes, such as biocontrol agents and plant growth-promoting rhizobacteria (PGPR), can suppress disease or enhance plant growth. Unlike *Rhizobium*, most non-pathogenic plant microbes do not invade the plant tissue. Many studies have shown plants differ in the level of disease suppression obtained from a particular biocontrol agent (King & Parke 1993; Liu et al 1995; Smith et al 1997). By using a tomato RIL mapping population, the host variation for disease suppression by the biocontrol agent *Bacillus cereus* UW85 was studied, and provided the first description of the interaction of plants with disease-suppressive bacteria (Smith et al 1999).

A method to study the role of host differentiation in disease suppression by a biocontrol agent is to characterize the host effects on biocontrol mechanisms (Smith et al 1999). Inducing host resistance is one of the mechanisms by which PGPR and other biocontrol agents.. Liu et al (1995) demonstrated cultivar specificity in the induced resistance to anthracnose of cucumber by PGPR strains. From another biocontrol mechanism perspective, plants can have a strong effect on microbial communities through antibiosis. For example, the production of 2, 4-diacetyle phloroglucinol (DAPG) by biocontrol strains is influenced by plants. The DAPG producers occur with lower

frequencies in non-rhizosphere soil than in the corresponding rhizosphere soil and are strongly affected by the cultivation of maize (Picard et al 2000). It was noted that the expression of 2, 4-DAPG in strain CHA0 was significantly lower in the rhizosphere of dicots (bean and cucumber) than in the rhizosphere of monocots (maize and wheat). It has been extensively reported in studies that the plant type can influence the genotypic and phenotypic diversity of bacterial strains producing secondary metabolites (Linda 2002; Garbeva 2004[a]; Raaijmakers 2002). Leonardo (2006) reported that the host crop plays a key role in modulating the ability of rhizosphere colonization by different genotypes of 2, 4-DAPG producing *P. fluorescence*. Population densities of indigenous 2, 4-DAPG producers differed in the rhizosphere of alfalfa, barley, beans, flax, lentils, lupines, oats, peas and wheat. Oats and lentils were measured to have the lowest and highest densities of 2, 4-DAPG producers respectively. It was found that permanent grassland and grassland-derived plots supported the highest densities of microbes producing the antifungal antibiotic pyrrolnitrin, while the prevalence of this pyrrolnitrin biosynthetic locus was found to be lower in the arable land plots as determined though real-time PCR assay (Garbeva et al 2004[a]).

Garbeva et al (2004[a]) explored the rhizosphere effect of maize, oats, barley and commercial grass on the abundance of bacterial antagonists of the potato pathogen *Rhizoctonia solani*, AG3. It was discovered that barley and oat rhizosphere supported the highest antagonistic *Pseudomonas* populations, compared to the rhizosphere of maize and grass. The proportion of isolates with antagonistic activity towards the soil-borne pathogen *Verticillium dahliae* was highest for the strawberry rhizosphere, followed by oilseed rape and potato rhizosphere. A rather high proportion of *Pseudomonas putida* B was identified from the strawberry rhizosphere, while *Enterobacteriaceae* (*Serratia* spp*., Pantoea*

agglomerans) were mainly isolated from the rhizosphere of oilseed rape (Berg et al 2002, 2006).

With the help of advanced culture-independent techniques, the relationship in plant-bacterial community interactions was revealed in more detail. The rhizosphere of canola was dominated by the α-Proteobacteria class and the Cytophaga -Flavobacterium-Bacteroides division (Kaiser et al 2001). There were four bacterial divisions in the maize roots, in which a new bacterial genus and species in the Flexibacter group, *Dyadobacter fermentens,* was found (Chelius & Triplett 2001). Haichar et al. (2008) reported plant root exudates shape soil bacterial community structure. Bacteria related to *Sphingobacteriales* were specific to monocotyledons such as wheat and maize, whereas bacteria of *Enterobacter*, *Rhizobiales* can colonize both monocotyledons and dicotyledons. A 2001 study found that old wheat cultivars were colonized by phylogenetically diverse rhizobacteria, whereas the rhizosphere of modern cultivars were dominated by fast-growing Proteobacteria (Germida and Siciliano 2001). In a pyrosequence-based study, Nacke et al (2011) found that different tree species had statistically significant effects on soil bacterial diversity, richness, and community composition in forests.

More and more evidence has shown that plants influence not only the structure of bacterial communities, but also bacterial function. Bremer et al (2007) reported that species of nonleguminous plants directly influenced the composition of *nirk*-type denitrifiers in the soil. It was also found that different rice cultivars shaped the composition and activity of ammonia-oxidizing bacterial population (Briones et al 2002).

2.1.4 The effect of soil on bacterial communities

Soil is a major determinant of the composition of microbial communities. Several soil traits such as texture, structure, organic matter, microaggregate stability, pH, salinity, and the presence of nutrients, determine the microbially habitable niches.

It is difficult to characterize the effect of soil on the changes in microbial communities by using culture dependent methods. It has been said that a "black box" was opened when microbial communities in soil were analyzed. However, with the advancement of modern technology and the development of culture independent methods, the microbial diversity in the soil under different environment disturbances has been described. By amplifying small subunit ribosomal DNA (SSU rDNA) from fractions with 63 and 35% G+C contents, Nusslein and Tiedje (1999) reported that the soil bacterial community composition differs in forest and pasture soil. *Fibrobacter* and *Syntrophomonas* are the most dominant members in forest soil while *Burkholderia* and *Rhizobium-Agrobacterium* assemblages dominate pasture soil.

Several related studies have provided evidence that soil is an important determinant of the structure of residing microbes. By analyzing the grouping of DGGE fingerprints obtained from soils from different geographical locations, Gelsomino et al (1999) found similar soil types tend to select similar communities. Chiarini et al (1998) reported that soil type had the greatest effect on the density and community structure of bacteria associated with the roots of field-grown maize, whereas different maize cultivars had no significant effect on these traits. Da Silva et al (2003) analyzed the diversity of *Paenibacillus* populations in maize plants grown in two different soils and obtained similar results showing that the soil type rather than the maize cultivar type was the determining factor that influenced the community structure of *Paenibacillus*.

In a study of Wisconsin pasture soil, three bacterial divisions, the Proteobacteria, the Fibrobacter, and the low G+C gram-positive bacteria, were represented in nearly 60% of the 16S rDNA clones (Borneman et al 1996). In Siberian tundra soil, it was found that over 60% of the 16S rDNA clones belonged to the Proteobacteria and 16% to the Fibrobacter division (Zhou et al 1997). It was also discovered that the soil type was the dominant factor influencing the diversity of the population of culturable fluorescent *Pseudomonas* spp. (Latour et al 1996). 2,4 -DAPG producers of the D genotype have been isolated from monoculture wheat fields throughout the United States, but have not been reported in European soils. In the Dutch soils studied to date, only genotypes such as F and M have been detected (De Souza & Raaijmakers, unpublished). Similar studies in Manitoba, Canada have revealed similar findings (Fernando and Li Ru, unpublished).

The frequency and relative proportion of specific clay minerals in soils may influence the activity of microbes. For example, the presence of iolite inhibited the antagonistic activity of fluorescent *Pseudomonas* spp. isolates against the pathogen *Thielaviopsis basicola*, whereas, this activity was stimulated by vermiculite (Stotzky and Post 1967; Stutzet al 1986[a, b]). This phenomenon seems due to the clay's interference with the iron uptake by the microbe. Fe^{3+} concentration is limited in alkaline and neutral soils and increases with increasing soil acidity. In this regard, siderophore-producing bacteria, with high affinities for iron, have been found to inhibit some iron requiring pathogens in alkaline and neutral soils (Schroth and Hancock, 1982; Dowling et al 1996).

Fierer and Jackson (2006) found that the diversity and richness of soil bacterial communities were highly correlated to soil pH with the highest bacterial diversity in neutral soils and lower diversity in acidic soils in a comparison of 98 soil samples from across North and South America. Salinity was found to be the major environmental determinant of

microbial community composition, based on comprehensive analysis of 21,752 16S rRNA sequences compiled from 111 studies of diverse physical environments (Lozupone and Knight 2007). In a field study carried out in Scotland, it was found that bacterial community structure was mainly driven by sample field location, and moisture had a comparatively higher impact on bacterial communities compared to soil nitrogen or carbon (Singh et al 2009).

Soil particle size also affects the diversity of microbes. Ranjard et al (2000) first demonstrated that microbial diversity varies with soil particle size by using ribosomal intergenic spacer analysis (RISA). Sessitsch et al (2001) found that bacterial diversity increase with decreasing particle size.α-*Proteobacteria* dominated in larger particle soils, while the Holophaga /*Acidobacterium* were common in clay particle soils. In addition, the soil with organic matter supported a greater microbial biomass.

2.1.5 The influence of agricultural management systems on bacteria

Agricultural management includes plant community structure, and the use of tillage, rotation, fertilizer, and fumigants. Compared to non-agriculture soil, such as forests and grasslands, bacterial communities can change in response to agricultural management practices (McCaig et al 1999, Acostamartinez et al 2008, Jangid et al 2008, Lauber et al 2008). Buckley and Schmidt (2001) explored the dynamic changes of microbial structure in response to different agricultural management practices. They found significant differences between a field used for long-term study of agricultural management (HCS) and a never cultivated succession field (NCS). Compared to HCS, alpha Proteobacteria, the beta Proteobacteria, and the Actinobacteria were significantly higher in the NCS field. In the western Amazon soil survey, it was found that changes in land use altered the structure of

bacteria. Bacteroidetes and Alphaproteobacteria were mostly found in crop and pasture soil, whereas Firmicutes and Actinobacteria were mostly found in the primary forest and old secondary forest (da C Jesus et al 2009).

2.1.5.1 Crop rotation, tillage and their influence on microbes

Crop rotation implies a temporal sequence in which different crops are grown on the same land (Las 2003). Crop rotation, which is now the most important cultural practice used to control of plant pathogens worldwide, is believed to improve yield and to reduce disease because it encourages the development of niche heterogeneity in the rhizosphere, and rotates with non-host crops. In contrast, monoculture, the repetitive growing of the same crop in the same land (Andrews and Kassam 1976) reduces microbial competition in the rhizosphere by lowering biodiversity among root-associated bacteria and fungi, thus enabling the pathogen population to develop (Knops et al 1991; Andrews and Harris 2000). Acosta-martinez (2008) reported that cotton-wheat-corn rotation supported higher bacterial diversity than cotton monoculture. In addition, *Thauera* was a unique bacterium in soil under cotton-wheat-corn rotation, while *Azosprillium* and *Prevotella* spp. were unique to the soil under cotton monoculture. By using T-RFLP and pyrosequencing, it was found that a wheat-oat-canola-pea crop rotation increases Proteobacteria significantly, compared to wheat monoculture (Li et al 2010[a,b]).

Tillage is the mechanical manipulation of the soil and the plant residue to prepare a seedbed where crop seeds can be planted. It directly influences the physical and chemical properties of the soil, affects the soil temperature and moisture, enhances the release of soil nutrients for crop growth, controls weeds and it is the major tool in soil sanitation by accelerating the decomposition of pathogens in crop residue. Intensive tillage can cause

soil erosion, accelerate soil carbon loss, increase greenhouse gas emissions, and creat unsuitable conditions for beneficial microbes that control plant pathogens. In response, farmers have shifted from conventional tillage to conservation tillage systems in many areas of the world.

According to the definition provided by the Conservation Technology Information Center (CTIC, 1995), conventional tillage includes "the tillage methods that leave less than 15% residue uncovered after planting, or less than 560kg $ha^{-1}$of small grain residue equivalent throughout the critical wind erosion period", while conservation tillage refer to "tillage methods that leave more than 15% crop residue (trash), or more than 560kg ha^{-1} of small grain residue on the surface of the soil (including minimum tillage methods)".

Different combinations of crop rotation and tillage practices could lead to various effects on changes in structure of microbial communities. Lupwayi et al (1998) investigated the effect of tillage and crop rotation on the diversity and community structure of bacteria by using the BIOLOG system. These studies revealed that conservation tillage and legume-based crop rotations support higher diversity of soil bacterial communities.

Disease incidence in monoculture corn in the central United States was studied under various types of reduced tillage and conservation tillage systems. Several foliar pathogens of corn, such as *Helminthosporium turcium, H. maydis, Phyllosticta maydis, and Cercospora zeae-maydis* were more severe in reduced tillage than in conservation tillage (Arny et al 1970; Burns and Shurtleff 1973; Hilty et al 1979; Boosalis et al 1981), while *Colletotrichum graminicola* could not be controlled in both tillage practices (Phillips et al 1980). Conservational tillage decreases soil temperature in the spring and early summer, and may be conducive to damping-off and diseases caused by soil borne pathogens favored by low temperature.

When the frequency of potato cropping increases, potato yields decrease due to the deleterious microbial communities. It has also been noted that for wheat, barley, bean and maize, the yields decrease with an increase in the frequency of cropping (Schippers et al 1985, 1986[a]). The deleterious effects are likely due to microbial metabolites secreted by pathogens, which affect physiological processes in root cells. There is evidence that accumulating cyanide inhibits the energy metabolism of potato root cells in high-frequency potato cropping, possibly owing to the accumulation of the HCN precursor in soil. The plant cultivars may differ in their sensitivity to the deleterious effect, therefore, cultivar rotation could be considered as a means to control pathogen and reduce yield loss.

However, there is a fairly well established phenomenon where the disease incidence decreases in long-term monoculture soil. For instance, it was found that take-all disease, caused by *Gaeumannomyces gaminis* var. *tritici*, decreased naturally in wheat monoculture fields, while the population of beneficial *Pseudomonas* spp. increased (Raaijmakers et al 1997, 1998, 1999). This type of soil is called a disease-suppressive soil. Many studies have discussed how disease-suppressive soils occur and the mechanisms involved (Mazzola 1999, Smith et al 1999, Parke & Gurian-Sherman 2001).

Conservation tillage and conventional tillage practices may increase, decrease, or have no effect on plant diseases. Ibekwe et al (2002) found biomass amounts were significantly higher and ammonia oxidizers more diverse in the no-till (NT) soil than in the conventional-till (CT) soil. However, by using phospholipid fatty acid analysis (PLFA) and denaturing gradient gel electrophoresis (DGGE), Helgason et al (2010) investigated long-term no-till management effects on microbial communities in the Canadian prairie agro-ecosystems. They found that differences in bacterial communities were related to depth in the soil profile rather than tillage management practices. Wortmann et al (2008) also found

that bacterial communities in the 0- to 30- cm depth, on a mass equivalent basis, were not significantly affected by tillage. It was found that richness and diversity of bacterial communities were slightly higher under zero tillage treatment, while the percentage of the majority of individual bacteria was similar under different tillage systems, except for Firmicutes and Chloroflexi, which showed different trends at $P = 0.1$ when using high throughput methods such as TRFLP and Pyrosequencing (Li et al 2010[a,b]).

Therefore, tillage practices should be chosen according to climate, vegetation history and disease incidence. More research is needed to understand the influence of different crops and specific tillage practices on plant diseases.

2.1.5.2 The effect of pesticides and fumigants

Pesticide has been widely used in conventional farming systems as a part of the pest control strategy. Globally, about $3x\ 10^9$ kg of pesticides is applied annually (Pan-UK, 2003). However, only 0.1% applied pesticides can reach target organisms while the remaining amount contaminates the soil environment (Carriger et al 2006). Many studies have showed that pesticides adversely affect the diversity and activities of beneficial soil bacterial due to their xenobiotic characteristics (Niewiadomska and Klama 2004; Wang et al. 2006, Widenfalk et al., 2008). There are also reports elucidating the ability of soil microorganisms to degrade pesticides and use applied pesticides as a source of energy and nutrients. These pesticides result in increasing the population sizes and activity of pesticide-degrading soil microorganisms (Das and Mukherjee 2000, Kumar and Philip 2006, Hussain et al 2007).

However, some studies also showed no adverse side effects of pesticides on microbial communities (Sarnaik et al 2006). Lupwayi et al (2009) investigated the effect of

the pesticides vinclozolin and cyhalothrin on functional soil bacterial diversity and function, and detected no side effects in canola rhizosphere or bulk soil. There are some cases that show that microbial populations initially affected by pesticide application acclimatize after a period of time, and return to normal (Filessbach and Mader 2004, Niewiadomska 2005).

The effect of three phenyl urea herbicides on microbial communities was examined by El Fantroussi et al (1999). The number of culturable heterotrophic bacteria decreased significantly with all of three herbicides. The decline of unculturable *Acidobacterium* was also observed. With an increase of bromoxynil concentrations, changes in the bacterial community were measurable. The addition of bromoxynil had a negative impact on the presence of α & γ-Proteobacteria in the soil (Baxter and Cummings 2006). Khan et al (2006) found that atrazine and isoproturon adversely affected *Bradyrhizobium* spp. It was found that mefenoxam and metalaxyl inhibited N-fixing bacteria (Monkiedje et al 2002).

However, there is some conflicting research on the effects of herbicides on soil bacterial diversity. Xia e al (1995) found no changes in the bacterial community in response to the application of 2, 4-dichlorophenoxyacetic acid (2, 4-D) at the recommended application rates. In contrast, Gonod et al (2006) revealed that the genetic structure of bacterial communities was significantly shifted in response to 2,4-D application during the intense phase of 2,4-D biodegradation. Busse et al (2001) showed glyphosate caused the decline of bacterial numbers in a pine plantation. In contrast, Nicholson & Hirsch (1998) reported an increase in cultural bacterial populations in soils treated with glyphosate.

Fumigants are widely used when farming high-value cash crops to effectively control nematodes, soil borne pathogens, and weeds. Ibekwe et al (2001) examined the changes in soil microbial community structure in a microcosm experiment following the

application of methyl bromide (MeBr), methyl isothiocyanate, 1,3-dichloropropene (1,3-D), and chloropicrin. They found high diversity indices were maintained between the control soil and the fumigant-treated soil, except for MeBr, where MeBr has the greatest impact on soil microbial communities and 1, 3-D has the least impact.

2.1.5.3 Fertilization

Fertilization is an important agricultural practice used to enhance plant nutrition and achieve high yield. Fertilizers can be classified as either organic (composed of enriched organic matter e.g. plant or animal) or inorganic fertilizer (composed of synthetic chemical or mineral). Many studies have investigated that effect of long-term fertilization on the soil fertility, organic matter, soil physical properties and crop yield (Pernes-Debuyers and Tessier 2004; Mallarino and Borges 2006; Cai and Qin 2006). Since microbial populations play an important role in plant residue decomposition and nutrient cycling, it is of interest to study how fertilizers affect the structure and function of microbes. By using PCR-DGGE and PLFA profiling, Clegg et al (2003) found nitrogen fertilizer has a significant impact on the total bacterial and actinomycete community structures. It was also found that fertilizer amendment altered the abundance of bacterial groups throughout the agriculture soil in Watkisville, Georgia by using both 16S rRNA gene clone libraries and phospholipid fatty acid (PLFA) analysis (Jangid et al 2008). One study showed that γ-Proteobacteria were sensitive to fertilization while Acidobacteria was unaffected by fertilization (Wu et al 2011). It was found that long-term inorganic nitrogen management influenced the structure of nitrite-oxidizing bacteria and new ecotype of no characterized *Nitrospria* spp. was found in fertilized soil (Freitag et al 2005). By using combination of qPCR, T-RFLP, cloning and

sequencing, Chen et al (2010) found that *nirK* containing denitrifiers were more sensitive to fertilization than *nirS* containing denitrifiers in a paddy soil.

There is a debate in the literature on which type of fertilizer is beneficial to the composition and function of microbial populations in the soil (Chu et al 2007; Esperschutz et al 2007; Shen et al 2010; Kamaa et al 2011). A long-term field experiment in Switzerland using the ester linked phospholipid fatty acid method found that long-term organic fertilization was strongly associated with the slow growing bacteria known as k-strategists (Esperschutz et al 2007). Shen et al (2010) compared the soil bacterial community size and structure under different fertilization treatments including no fertilization (CK), inorganic-N fertilization (N), organic manure amendment (M) and half N-fertilizer plus half organic manure (M+N). They found that the bacterial community under N treatment was significantly different from the other treatments. The N treatment had the smallest size of the general bacterial population and was lacking any Gammaproteobacteria. Similarly the Kabete long-term trial in Kenya revealed that bacterial community structure and diversity was negatively affected by inorganic NP fertilizer, and that microbial community in the soil with organic input was clustered away from the soil with inorganic input (Kamaa et al 2011). Chu et al (2007) found organic manure fertilizer promoted the population of *Bacillus* spp. in soil, compared to inorganic fertilizer. In contrast, Chen et al (2010) found organic and inorganic fertilizer had similar effects on the composition of soil denitrifying communities.

The interaction of fertilizers and pesticides effects microbial diversity and activity in the soil. It was reported that the structure of microbial communities is influenced by the co-treatment of inorganic fertilizer (KH_2PO_4 and NH_4HCO_3) and pesticides (Acetochlor, Fenvalerate, Thiophanate-Methyl) (Chen et al 2006).

2.2 Methods to study microbial communities in soils

The methods to study microbial communities in soils can be categorized into two groups: biochemical-based methods and molecular-based methods. Biochemical-based methods include plate counts (Sands et al. 1980), protein analysis (De Vos et al. 1993), comparing substrate utilization (Garland and Mills 1991; Grimont et al 1996), and fatty acid methyl ester (FAME) profiling (Stead 1992; Vancanneyt et al 1996). Molecular-based methods consist of measuring nucleic acid reassociation kinetics (Torsvik et al 1990, 1996), Guanine plus cytosine (G+C) content (Nusslein and Tiedje 1999), DNA microarrays and DNA hybridization (Cho and Tiedje 2001), DNA cloning and sequencing (Moore et al 1996), DNA and RNA hybridization (Amann et al 1996), and pyrosequencing (Roesch et al 2007). Other PCR-based "finger printer" methods include terminal restriction fragment length polymorphism (T-RFLP) (Liu et al 1997), denaturing gradient gel electrophoresis (DGGE) (Myers et al 1985), temperature gradient gel electrophoresis (TGGE), ribosomal intergenic spacer analysis (RISA) (Ranjard et al 2001). Every method has its advantages and disadvantages.

2.2.1 Biochemical-based methods

Plate counting is an isolate-based method, which characterizes carbon source utilization providing a useful means to understand of the growth habit, development, and potential function of microorganisms from the soil environment. In addition, it has the advantage of providing rapid results as well as being reproducible and inexpensive. However, because of the need for isolation and the cultivation of individual species, these methods are biased towards fast growing microorganisms and cannot detect unculturable

microorganisms. Therefore, the diversity of microbes in environmental samples is underestimated.

The substrate utilization method is based on tetrazolium dye reduction as an indicator of sole-carbon source utilization to profile the physiological requirements of a microbial community. Biolog systems (Biolog GN Microplates and Ecoplates) were widely used to determine how microbial communities respond to various factors including soil type (Miethling et al 2000) plant species (Fang et al 2001; Siciliano et al 1998), and agricultural managements (Larkin 2003; Lupwayi et al 1998). Although this method is able to differentiate microbial communities at the community-level using physiological profiling, it bears some fundamental problems. Primarily, it is still based on the culture-dependent method to assess microbial communities, and favors rapidly growing microbial species that can utilize the carbon substrate. Therefore it is unlikely that this method provides a more comprehensive understanding of microbes on the communities level when compared with other media-based culture methods. Furthermore, since the BIOLOG system analyzes the metabolic reaction pattern, it is unable to distinguish which microbial communities are responsible for carbon source utilization.

Fatty acid methyl ester (FAME) profiling is other widely used bio-chemical method to assess the structure of microbial populations based on detection of the fatty acids, a relatively constant component of the cell membranes which can be used to differentiate major taxonomic groups within a community (Ibekwe and Kennedy 1998). This method can differentiate the active microbial biomass and the non-living microbial biomass. Despite the advantages of this method, there are some limitations. Cellular fatty acid can be influenced by factors such as temperature and nutrition.

2.2.2 Molecular-based methods

It has been estimated that there are nearly 10^7 microbial species in one gram of soil (Gans et al 2005). However, less than 1% of these microorganisms can be cultured, and so the majority of them are in the "black box". Molecular-based methods provide an invaluable opportunity for us to expand our knowledge of soil microbial communities. They have also been used to monitor how different plant, soil; agricultural management practices influence soil microbial communities. Among the numerous tools available, approaches based on the amplification of the target DNA or RNA sequence extracted from soil will be addressed. The 16S rRNA genes of bacteria or archea are most commonly targeted and amplified because they possess highly conserved and hypervariable regions. They are used for examining particular organisms or taxa of interest through universal or taxa specific primers. In the past decade, the database of 16s rRNA gene sequences has grown to 2,639,157 (Ribosomal Database Project, 2012), enabling us to differentiate more microbes and discover greater diversity in microbial communities.

2.2.2.1 Quantitative Real-Time PCR

Real-time PCR or quantitative PCR (qPCR) is used to amplify and simultaneously quantify a target DNA. Unlike normal PCR, qPCR detects the PCR products at each cycle during the exponential reaction instead of at the final phase or the end point of the reaction. This allows researchers to view the accumulation of amplicons during the reaction, and therefore the amplicons can be quantified more accurately. It can be used to estimate the changes in a specific species or genus of microorganisms (using specific primers) (Dang et al 2010; Szukics et al 2010; Davis et al 2009), or shifts in microbial communities (Hollister et al 2010; Andreote et al 2009; Wakelin et al 2009). Quantitative PCR is becoming a

useful tool to evaluate dynamic changes of microbial communities in complex soil environments.

2.2.2.2 Cloning libraries and sequencing

Using this approach, microbial population can be identified by PCR amplification of 16S ribosomal genes, and subsequently cloning and sequencing. The microbial community composition and the species richness and abundance can be compared to the sequence information in sample libraries (Borneman et al 1996; Kielak et al 2008; Dimitriu et al 2010). Since the average size of clone libraries constructed is only 100-500, abundant microbial communities will be identified and rare species of ecosystems usually will not be detected. Therefore, in a survey of highly diverse ecosystems, this method may result in an undervaluation of species richness (Noha et al 2009).

2.2.2.3 PCR-based fingerprinting methods

Since the strength of fingerprinting techniques lies in the fact that large amounts of samples can be analyzed and compared, they represent facile tools to assess the composition and structure of microbial communities in certain assemblage, or to assess microbial changes between the samples or treatments.

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are two widely used tools based on the similar principle. The theory of DGGE and TGGE is that the two similarly sized DNA fragments differing in a single base can be separated from each other by passing through an acrylamide gel employing either a chemical gradient (DGGE) or temperature gradient (TGGE) (Myers et al 1985). Subsequently, DNA fragments form multiple bands that are used to calculate the richness, diversity, and similarity index of microbial communities in samples. The

differences in the banding patterns can be used to differentiate the genotype of specific microbial taxa (Smalla et al 2007; Freitag et al 2005; Raaijmakers et al 2002). Furthermore, specific DGGE bands can be excised from gels, re-amplified, sequenced or transferred to membranes, and hybridized with specific primers to obtain information about the specific taxonomic groups within the community (Chong et al 2009). With the use of internal standards both during DNA extraction and the PCR-DGGE procedure, this method can even be used to estimate relative abundance of diversity (Petersen et al 2005). This method has the advantage of being reproducible, rapid and reliable. Furthermore, a large number of samples can be analyzed simultaneously. Meanwhile, the limitations of DGGE include PCR biases, only dominant species being detected and co-migration means one band can represent more than one species (Jennifer et al 2004).

Random amplified polymorphic DNA (RAPD) is another fingerprinting tool for studying microbial diversity. Instead of amplifying the 16S ribosomal gene, it works by amplifying random genomic sequences. This method requires no prior genetic information for samples. RAPD randomly amplifies the segments of DNA using short primers (8-12 nucleotides). Normally, several primers are used and the one, which yields the best discrimination of microbial communities, is chosen (Denman et al 2005). The drawbacks of RAPD are that they are very laborious, not reproducible, and the results are difficult to interpret.

Ribosomal intergenic spacer analysis (RISA) is a method in which the rRNA gene operons of the intergenic spacer region (so-called ISR) between 16S rRNA and 23S rRNA are amplified by using primers containing conserved regions of the 16S and 23S genes. The strength of RISA lies in its ability to generate significant length heterogeneity in the ISR (range between 150-1500 bp) (Ranjard et al 2001; Borneman et al 1997), and to

discriminate closely related strains (Denman et al 2005). It has been used to construct bacterial community structures associated with different perturbed soils including metal pollution (Ranjard et al 2000), antibiotic treatments (Robleto et al 1998), and different vegetation covers (Borneman & Triplett 1997). Restriction fragment length polymorphism (RFLP), a technique involving the amplification of total bacterial DNA and digestion of resulting PCR products, is a rapid screening method that presents the ribosomal diversity of microbial populations (Denman et al 2005).

Terminal restriction fragment length polymorphism (T-RFLP) and automated ribosomal intergenic spacer analysis (ARISA) are two methods which have been developed to improve the detection and resolution of fragment analysis by utilizing fluorescently labeled oligonucleotide primers for PCR amplification and automated systems for separation and detection of PCR fragments. Compared to methods that use standard gel electrophoresis detection, automated electrophoresis systems offer high throughput and rapid analysis of microbial community structure. In addition, the high sensitivity and precision of fluorescence detection increases the number of peaks detected, and allows for a more accurate comparison of community profiles based on the band intensity.

T-RFLP primarily amplifies small subunit (16S or 18S) rRNA genes from the entire microbial community using PCR with fluorescently dye labeled primers. The resultant PCR products are then digested with restriction enzymes, which have 4 or 6 base-pair recognition sites. Only terminal restriction fragments (T-RFs) tagged with fluorescently labeled dyes can be automatically checked, and their relative abundance and size are measured by a DNA sequencer or specialized T-RFLP machine. Since the different lengths and patterns of T-RFs reflects the difference in sequence of 16S or 18S rRNA, the structure and composition of microbial communities can be determined. One advantage of T-RFLP

is that it is the only method "offering phylogenetic information directly without further sequencing of the fragments compared to other fragment analysis methods" (Kent & Triplett 2002). Because it is very cost-effective and relatively simple, T-RFLP has been widely used to assess changes in the structure and composition of various microbial communities residing in different environments including bacteria, archae, and fungi (Yan et al 2009; Pérez-Piqueres et al 2006; Wu et al 2006; Nabla et al 2005; Kennedey et al 2005; Leybo et al 2006; Johnson et al 2004; Sessitsch et al 2001; Lukow et al 2000; Liu et al 1997). Additionally, T-RFLP has been used to analyze functional genes including methane oxidation (Mohanty et al 2006; Horz et al 2000 & 2001) and nitrogen fixation (Rosch et al 2006; Tan et al 2003). However, since multiple copies of rRNA and other functional genes exist in microbial species, T-RFLP results are both difficult to adjust for and can result in bias. In addition, incomplete digestion by restriction enzymes could also lead to an overestimation of diversity (Osborn et al 2000).

2.2.2.4 Pyrosequencing

Pyrosequencing, a new high-throughput sequencing technique, has become a powerful approach in 16S gene-based microbial diversity surveys. Pyrosequencing technology is based on detection of the released inorganic pyrophosphate during DNA synthesis. The general principle of a pyrosequencing reaction is that a pyrophosphate (PPi) is released and subsequently converted to ATP by ATP sulfurylase when nucleotides are incorporated into a nucleic acid chain by DNA polymerase. The ATP provides the energy to luciferase to oxidize luciferin and generate light, which can be detected by a photodiode, photomultiplier tube, or a charge-coupled device camera (CCD). By choosing proper primers and robust community comparison methods, and reassembling 100-400 nucleotides

of rRNA sequences produced by pyrosequencing, it is possible to taxonomically cluster different microbial communities at the genus level with high accuracy (Roesch 2009; Liu et al 2007). The advantages of this approach are: (1) rare members of the microbial community can be detected even with inadequate sampling since a large number of 16S rRNA gene sequences are produced (e.g. Roche 454 GSXLF pyrosequencing machine can produce 400Mb per run) (Noha et al 2009); and (2) the mixed DNA samples can be sequenced without constructing clone libraries. Current bioinformatics tools are outstripped by the abundance of data generated by next generation sequencing technologies representing a bottleneck for pyrosequencing and leading to problems using and interpreting the data (Hugenholtz & Tyson 2008). Currently, this technique is used to assess highly diverse microbial communities inhabiting the soil (Roesch et al 2007; Kim et al 2008; Teixeira et al 2010).

2.2.2.5 Metagenome

"Soil is the most biodiverse environment on the earth: it is estimated to contain approximately 1,000 Gbp of microbial genome sequences per gram of soil! Compared with the Human Genome project (in which 3 Gbp were sequenced)" (Vogel et al 2009). Soil sequencing is in contention to be the next major global metagenomics initiative since the soil metagenome would lead to new environmental breakthroughs and economic opportunities. Recently high-throughput sequencing methods have provided powerful tools to interpret the soil metagenome. Large-scale metagenomic sequencing efforts will provide sufficient data to understand soil microbial community diversity and function under different perturbations and their resilience capability, which will then provide insight into the ecology of microorganisms that are beneficial to, or threaten, crop production. The

launched soil metagenomic project, TerraGenome International Sequencing Consortium, will contribute significantly to the goal of sustainable agriculture.

Owing to the biodiversity of soil microbes and the limitation of analytical techniques, it is still unclear how different cropping systems influence the diversity and function of soil microbes. As mentioned above, with the help of advanced molecular and next generation sequencing technology, it is possible to study nonculturable microbes dwelling in soil. Meawhile, informative evidence for advocating low –input agriculture needs more data through research in terms of health of soil biology traits. Therefore, based on previous studies, we used both culturable and molecular methods to test our hypothesis: (1) The bacterial community structure, could be influenced by different cropping systems. As such, crop rotation, zero tillage and organic farming systems could increase the richness, and diversity of bacteria, compared to cropping system with monoculture, conventional tillage, and conventional farming systems; (2) The antibiotic-producing *Pseudomonas* populations would be influenced by different cropping systems.

3.0 INTEGRATING MOLECULAR-METHODS AND CULTURE-BASED METHODS TO COMPREHENSIVELY EXPLORE BACTERIAL COMMUNITIES THAT INFLUENCE PLANT HEALTH UNDER DIFFERENT CROPPING SYSTEMS

3.1 Abstract

By using culture-dependent and molecular methods, diversity and richness of soil- and root- associated bacterial communities and the population of antibiotic-producing Pseudomonads was assessed under different cropping systems in Manitoba, Canada. These systems included canola monoculture (CM), wheat monoculture (WM), wheat and canola rotation (WR or CR), zero tillage (ZT), conventional tillage (CT), Grain-only organic (GO), Grain-only conventional (GC), Forage-grain organic (FO) and Forage-grain conventional (FC). Both methods revealed that the cropping systems influenced diversity, richness of bacterial communities and frequency of antibiotic-producing pseudomonads to varying degrees. Culturable methods and tools of specific PCR-primer screening antibiotic genes revealed that zero tillage and organic farming systems supported a higher number of potential biocontrol *Pseudomonas* strains. Interestingly, world-wide distribution of *Pseudomonas* strains, which produce 2,4-diacetylphloroglucinol (2,4-DAPG) and pyoluteorin, were not found in all of the soil samples, whereas the frequency of pyrrolnitrin (Prn) and phenazine-1-carboxylic acid (PCA) producing *Pseudomonas* strains were higher in organic, ZT and canola associated soil samples. By using TRFLP, it was found that bacterial communities showed significantly different populations between canola and wheat. It was also demonstrated that organic and conventional farming practices exerted a major role in shaping the bacterial consortia.

3.2 Introduction

Bacterial communities are one of the major drivers maintaining physical, chemical and biological structure and function of the soil. Bacterial communities and involved in many ecosystem processes such as forming and restructuring of the soil matrix, cycling nutrients, decomposing organic matter, fixing N_2 and removing toxin (Preston et al 2001; Groffman et al 1986). Particular bacterial species in soil, mostly belonging to *Pseudomonas* and *Bacillus* spp. prevent infectious diseases of plants through different mechanisms including producing antifungal antibiotics and volatiles, eliciting induced systemic resistance in the host plant, and directly promoting plant growth (Haas & Défago 2005; Kloepper et al 2004; Weller et al 2002; Mazzola 1999).

Pseudomonas is widely distributed in soils from diverse geographical regions (Raaijmakers et al 1997; Keel et al 1996) and easy to be recovered on laboratory media. Pseudomonads produce versatile antibiotics including pyrrolnitrin (Prn), phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (2,4-DAPG) and pyoluteorin (Plt). These antibiotics have been shown to play an important role in suppression of plant diseases in many cropping systems. Therefore, some strains of *Pseudomonas* spp. are promising biocontrol agents (Raaijmakers et al 2002; Haas & Défago 2005). The natural distribution of antibiotic-producing bacteria was traditionally isolated, identified and characterized, which was time-consuming. With the help of advanced molecular tools (specific primers and probes) developed over the last few decades, the detection of natural strains of bacteria harboring antibiotic producing genes has provided a more expeditious approach as compared to the random isolation and screening procedures (Raaijmakers et al 1997; Garbeva et al 2004[a,b]). Some studies showed that specific genes within the biosynthetic loci of PCA, 2,4-DPAG, Prn were conserved among various *Pseudomonas*

strains worldwide, and that the prevalence of these antibiotic producing loci in soil could be affected by different soil treatments (Raaijmakers et al 1997; Garbeva et al 2004[a,b]; Svercel et al 2010). However, so far little is known about how different cropping systems influence the prevalence and diversity of the indigenous antibiotic-producing bacteria in soil.

Recent research has shown that the way used to manage soil and crops strongly influences the structure and function of bacterial communities dwelling in soil. However, the effects of agriculture disturbances on soil bacterial diversity have not been fully understood (Kennedy 1999). This is important since soil is one of the largest reservoirs for microorganism-nearly 10^7 microbial species per gram soil (Gans et al 2005).

Bacterial diversity and function can be analyzed using culture and molecular methods. Culture-based methods provide useful information about the growth habit, development, and potential function of bacteria from the soil environment. In addition, they have the advantage of providing fast results, being reproducible and inexpensive. These techniques are limited because less than 1% bacteria can be cultured in the laboratory (Torsvik et al 2002).

The use of molecular methods has broadened our knowledge about unculturable bacteria (Zhou et al 2004; Janssen 2006; Smalla and van Elsas 2010). Terminal restriction fragment length polymorphism (T-RFLP) is one high throughput molecular method, which has been shown to be effective at discriminating microbial communities in a range of environments (Blackwood et al 2003; Kennedy et al 2005; Genney et al 2006; Hullar et al 2006; Singh et al 2006; Leybo et al 2006). It amplifies a portion of the 16S rRNA gene from total bacterial DNA using fluorescently labeled primers followed by restriction digestion and detection of terminal restriction fragment (T-RF) through a capillary electrophoresis.

Over the last century, agriculture has been characterized are employing monoculture, tillage, chemical fertilizers and pesticides, which lead to increasing productivity of crops. However, this type of agriculture has resulted in numerous environmental issues such as fertilizer and pesticide leaching, underground water pollution, and decrease of ecological diversity (Mader et al 2002). In addition, the soil quality has been deteriorated which could directly affect the health and productivity of crops. Therefore, the objectives of this study are: (1) to use cultural methods and specific primers to detect antibiotic genes to assess culturable bacterial communities and *Pseudomonas* population shifts under different agricultural systems; (2) to use T-RFLP to analyze whole bacterial community changes under different agriculture systems in Manitoba, Canada; (3) to answer some ambiguous questions regarding whether ZT, crop rotation and low input organic farming systems are beneficial to bacterial community diversity and function, compared to conventional tillage, monoculture and conventional farming systems.

3.3 Materials and Methods

3.3.1 Sample site description and experimental design

3.3.1.1 Ian N. Morrison Research Station Site

Wheat, canola monoculture trial, wheat-canola-oat-pea rotation trial, and ZT, conventional tillage trials were set in 2003 at the Inn Morrison Research Station, which is located in Carman, 70 km southwest of Winnipeg, Manitoba, Canada. The soil type was a Rignold loam. A randomized complete block design with three replicates was used for the monoculture and rotation trials. Conventional and ZT practices were conducted on the canola monoculture trial. Conventional tillage was carried out using a deep tiller with tine

harrow after canola was harvested in the fall of 2005 and using a cultivar with tine harrow and coil packer in the spring of 2006.

3.3.1.2 Glenlea Research Station Site

Two Field trials were set in Glenlea Research Station, Winnipeg, Manitoba, Canada. The Glenlea Research Station is located 20 km south of Winnipeg, (Lat/Long. N 49, 39, 0 / W 97, 7, 0). The soil type is a Rego Black Chernozem comprising 12% sand, 32% silt, and 55% clay, with an organic matter content of 5.5%. The experimental design is a randomized complete block with 3 replicates. Two crop rotations including flax-oat-fababean-wheat (also called grain only rotation) and flax-alfalfa-alfalfa-wheat (also called grain forage rotation) were conducted under both certified organic and conventional methods and all rotation crops appeared in the rotation each year. No pesticides and chemical fertilizer were used in organic plots, and both pesticides and chemical fertilizers were used in conventional plots. The fababean served as green manure in the organic field while it was harvested as seed in the conventional one.

3.3.2 Soil sampling

For the Ian N. Morrison Research Station site, bulk soil was collected throughout the canola and wheat plots in May (before seeding) and in August 2006. Canola and wheat rhizosphere soil was sampled in August 2006. At the Glenlea Research Station, bulk soil and rhizosphere soil were sampled throughout the wheat and flax plots in June and August of 2008. All bulk soils were randomly collected from the soil top level (0-10cm) throughout the plots, followed by sieving (2mm) to remove stones and crop residues, and used for homogenization. Rhizosphere soil was collected from root systems after gently shaking off soil of loosely adhering to the plant roots. All samples were kept at -20°C.

3.3.3 Bacterial isolates, media and growth conditions

Five-gram bulk soil and rhizosphere soil samples were suspended in 45ml of 0.8% NaCl containing 5 g aquarium gravel (2-4 mm diameter), and shaken for 25 min at 250rpm. A 1-ml volume of suspension was prepared in serial 10-fold dilutions in 0.8% NaCl. A100 µl aliquot from the 10^{-4} and 10^{-5} dilutions was plated on 1/3 King's media B and Pseudomonas selective medium based on the Simon-Ridge medium (Simon and Ridge 1974) 1/3 KMB $^{+++}$ (consisting of 1/3 KMB supplemented with 40µg/ml ampicillin, 13µg/ml chloramphenicol and 100µg/ml cycloheximide) (Gardener et al 2001), and the plates were incubated at 27 °C for 48 h. Bacterial and Pseudomonas colonies on King's media B (KMB) and pseudomonas selective media were enumerated and results were presented as the log CFU per gram. A single colony was selected to ensure pure cultures. Pure cultures of each bacterium were stored in king's media B broth (KMB) amended with 18% glycerol (Fisher Scientific, Fair Lawn, NJ, U. S. A.) at -80°C.

3.3.4 Fungal strain (*Sclerotinia sclerotiorum* ss33) and culture conditions

Sclerotinia sclerotiorum ss33, which is a virulent strain, was used for all experiments. The fungus was routinely cultured on potato dextrose agar ((PDA) Difco Laboratories, Detroit, MI) media. The sterilized, cut sclerotia were placed on the middle of the PDA plate for 7-8 days at room temperature until the mycelia covered the entire surface of the plate.

3.3.5 Screening antibiotic-producing *Pseudomonas* strains *in vitro*

Antibiotic-producing *Pseudomonas* strains were screened using modified method described by Duncan et al (2006). Briefly, PDA media was used for testing the antifungal ability of bacteria against *S. sclerotiorum in vitro*. A loop of subcultured bacteria was inoculated in LB broth and incubated on the shaker for 16 h at 28 °C and 160 rpm. A volume of 5 µl of four different bacterial suspensions was then pipetted onto the PDA media equidistantly from the center of the Petri dish and incubated at room temperature for 24 hr. A 5 mm plug of freshly cultured *S. sclerotiorum* was placed on the middle of the plates inoculated with bacterial suspension. The mycelial growth of *S. sclerotiorum* and the numbers of sclerotia were measured everyday for 7 days. The *S. sclerotiorum* plug without bacteria challenge on the plate was used as a control. Bacterial inhibition activity was calculated by using the formula 100(R-R1)/R, where R is the radius of petri dish and R1 is the radius of mycelia growth of *S. sclerotiorum*. The inhibition test assays were repeated three times. The bacteria consistently showing fungal inhibition were stored in LB broth mixed with 20% glycerol, and kept at -80 °C.

3.3.6 Bacterial identification

Some of the bacteria showing stable antifungal activity was identified by using the Microlog^{TM} system (Biolog Inc., Hayward, Calif.). The purified bacteria were grown on Biolog universal growth (BUG) agar medium (Biolog Inc.,). Then Gram stain of single colony was performed. Bacterial numbers were estimated using a turbidimeter and 150 µl of the bacteria suspension was pipetted into a 96- well Biolog GN (Gram negative) or GP (Gram Positive) microplate. The plates were then incubated at 30 °C for 16-24 h. The plates

enabled identification to genus or species level using an automated plate reader (Biolog Inc.).

3.3.7 PCR-based screening of genes involved in biosynthesis of antibiotics

The isolated strains showing inhibitory activity were streaked onto KMB agar. One loop of bacterial culture was transferred to five milliliter of KMB broth and grown overnight. Total genomic DNA was extracted using cetyltrimethylammoniumbromide (CTAB)-based miniprep protocol (Ausubel et al 1995) with slight modifications. Two milliliter of bacterial suspension was transferred to a 1.5 ml sterile microcentrifuge tube, and was centrifuged at 7000 g for 10 min till a bacterial pellet formed at the bottom of the microcentrifuge tube. The bacterial pellet was resuspended in 567 µl of TE buffer. Three microliters of Proteinase K (20mg/ml) and 30 µl of 10% sodium dodecyl sulfate (SDS) were added into the bacterial suspension and mixed thoroughly, followed by incubation at 37 °C for 1h. Then 100 µl of NaCl was added to the suspension and mixed thoroughly, followed by 80 µl of CTAB/NaCl which was mixed again and incubated at 65 °C for 10 min. Next a is that 0.7 to 0.8 ml volume of chloroform/isoamy alcohol mixture (24:1) was added, mixed thoroughly and centrifuged at 7000 x g for 10 min. The supernatant was then transferred to a fresh tube, and an equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1) was added, mixed and centrifuged at 7000 x g for 10 min. The supernatant was transferred to a new tube, and mixed gently with 0.6 volume of isopropanol until a string of white DNA precipitate formed. The DNA was pelleted by centrifugation and washed twice with 70% ethanol. The final clean DNA pellet was dissolved in 100 µl of sterile de-ionized distilled water. The concentration of the DNA

samples was measured using a spectrophotometer and a 20 ng/µl DNA working solution was prepared.

PCR was used to detect the antibiotic biosynthetic genes for phenazine, pyrrolnitrin, pyoluteorin, and 2, 4-DAPG. The primer sequences, gene targets and references are listed in Table 3.1.

PCR amplification was conducted in a 25-µl reaction mixture containing 20 ng of template DNA, 1x PCR buffer, 1.5mM $MgCl_2$, 200 µM of each dATP, dCTP, dGTP, and dTTP, 20 pmol of each primer (Invitrogen Life Technologies, CA, USA), and 2.0 U of platinum®Taq (Invitrogen Life Technology, CA, USA). The PCR was carried out with a PTC-100™ programmable thermal controller. The list of PCR programs used for the amplification of antibiotic biosynthetic genes is presented in Table 3.2. PCR products were loaded onto a 1.5% agarose gel containing ethidium bromide and electrophoresed in 1x Tris-Borate EDTA (TBE) buffer at 100V for 45 min. The gels were visualized with a UV transilluminator and digitally recorded.

3.3.8 HPLC analysis to test for antifungal metabolites

Antifungal metabolites, found in the cell-surface extracts of bacterial strains that showed good inhibition zones as well as PCR products of the target gene, were analyzed using HPLC. For detection of Phenazine, 2, 4-DAPG by HPLC, 5 ml of the bacterial culture broth was extracted and analyzed following protocols described previously (Zhang et al 2006). In brief, bacterial strains were grown in potato dextrose broth, which favors production of phenazine and 2, 4-DAPG for 48 h on a rotary shaker. A 5 ml volume of cell-free culture supernatant was acidified to a pH of 2.0 with 56.5 µl of 10% trifluoroacetic acid (TFA). The resulting broth was extracted twice with ethyl acetate. The organic phase

was then evaporated to dryness and the residue was resuspended in 30% acetonitrile (ACN) + 0.1% TFA. Extract suspension was injected into a HPLC machine (EMD Chemicals Inc. An affiliate of Merck KGaA, Darmstadt, Germany). The HPLC system is composed of a Waters 2695 Separations Module, a Waters 996 photodiode Array Detector. Extracts were fractionated with a LichroCART® 250-4 RP-18 (5μm) cartridge at a flow rate of 1.0ml/min, using linear gradient of 10% ACN + 0.1% TFA as initial solvent conditions at 0 min, increasing ACN to 100% over 20 min. HPLC profiles were monitored with the photo diode array at 247nm (phenazine), 270nm (phloroglucinol). For pyrrolnitrin detection, bacteria were cultured in 5ml 523 media for 2 days at 27°C with 200 rpm (Kado and Heskett 1970). Pyrrolnitrin was extracted and analyzed following protocols described previously (Kraus and Lopper 1995). In brief, the cultured bacterial broth was centrifuged at 5000 x g for 5 min, and resulting bacteria pellet was suspended in 5 ml acetone. After the suspension was sonicated for 30 sec, it was centrifuged at 10,000 x g for 10 min, and the acetone supernatant was removed and dried. This acetone extract was adjusted to pH 2.0 with 1M HCl and extracted three times with 2 ml of ethyl acetate. The organic extraction phase was combined and dried, and finally dissolved in 100 μl of methanol. The sample was analyzed by C_{18} reverse-phase HPLC. Extracts were fractionated at a flow rate of 1.0ml/min, using 45% water/30% acetonitrile/10% methanol (vol/vol). Pyrrolnitrin was monitored with the photo diode array at 225nm.

3.3.9 Soil DNA extraction

Rhizosphere and bulk soil samples (0.25g wet weight) were washed prior to lysis to help remove inhibitors for downstream PCR, such as humic acid, covalent cations and other easily-dissolving organic compounds from the soil samples (He et al 2005). A 0.25 g soil

sample was mixed with 1.25 ml sodium phosphate (0.1 M, PH7.5), and the mixture was incubated in a shaker for 1 hr. at room temperature, followed by centrifuging for 10 min at 16000 x g. Supernatant were discarded. The DNA was then extracted from these pre-washed soil samples using the PowerSoil DNA isolate kit according to the manufacturer's specifications (Mobio Laboratories, Solana Beach, CA). The average DNA yield was 30ng/µl.

3.3.10 PCR amplification and T-RFLP

Bacterial composition of soil samples was estimated using T-RFLP as described by Sepehri et al (2007) with some modification. The V4 region of the 16S rRNA genes was amplified using Primers forward (5'-AYTGGGYDTAAAGNG-3') and reverse (5'-TACCRGGGTHTCTAATCC-3'). The forward primer was fluorescently labeled (WellRED D4 dye, Sigma-Proligo, St Louis, MO). The Polymerase Chain Reaction conditions were: (1) DNA was pre-heated for 5 min at 95°C, then the DNA was put on the ice immediately after heating; (2) the normal PCR process was conducted as follows: 95°C for 3 min, 35 cycles of 95°C denaturation for 30 sec; 56°C annealing for 45 sec; and 72°C extension for 1 min, followed by 72°C final extension for 5 min. The PCR products were cleaned to remove dNTP and Primers, and then digested with *HhaI* to produce terminal restriction fragments (T-RFS) (16.8 µl of PCR products, 10 units of *HhaI*, 1x *HhaI* buffer, and 20 µg of bovine serum; New England Biolabs, Ipswich, MA) at 37°C for 3 hrs. To obtain the precise lengths of the T-RF amplicons, the mixture of 5 µl fluorescently labeled fragment, 27 µl sample loading solution and 0.5 µl 400 bp DNA standard were loaded on the CEQ 8800 genetic analysis system (Beckman Coulter, Inc, Fullerton, CA). An

electropherogram with peaks of different sizes was obtained for each soil sample. Each peak of a different size represented a unique operational taxonomic unit (OUT).

3.3.11 Fragment analysis

The T-RFS was analyzed by using CEQ software (version 9.0; Beckman Coulter, Inc., Fullerton, CA). A binning parameter of 2 bp was used to construct the OTU profiles of T-RFLP data. A proportional threshold was used to detect signals from electronic noise (SchetÜtte et al 2008). Succinctly, the relative abundance of T-RF was calculated by dividing the height of each peak by the total peak height of that particular sample. Only peaks with relative abundances higher than 1% of the total were included in the analysis. The incidence (presence/absence) based data inferred from the OTU profiles were sorted based on different treatment, sampling time and soil type and used for numerical analysis.

3.3.12 Richness, diversity and similarity estimation

Chao1 incidence-based index of richness and Shannon incidence-based diversity index were performed with EstimateS (version 7.5; http//purl.oclc.org/estimates) to estimate the richness and diversity of microbial communities in each treatment, soil type/time group. 500 randomization of sample order was conducted for each test to reduce the effect of sample order. To determine rare or infrequent species, an upper abundance limit of 5 was used. The UPGMA clustering analysis based on similarity index Jaccard among microbial communities was performed using Paleo-ecology Statistics package PAST version 2.03 (http://folk.uio.no/ohammer/past).

3.3.13 Statistical analysis

The logarithmic transformation of bacteria and *Pseudomonas* counts under different treatments was analyzed using ANOVA, a mean separation test (Tukey's Least significant Difference) (P=0.05) using SAS version 9.2 (SAS Institute, Cary, NC, U.S.A.). To test significant differences in T-RF population structure between the different cropping systems, Multi-Response Permutation Procedures (MRPP) in Vegan package (R software) were performed.

3.4 Results

3.4.1 Fluctuation in total bacteria population and *Pseudomonas* populations under different cropping systems

The numbers of total bacterial populations and *Pseudomonas* populations growing on KMB and KMB+++ media are presented in Table 3.3 and Table 3.4. At Ian Morrison Station, there were significant effects of treatment, crop and tillage ($P<0.05$) on the total bacterial populations and *Pseudomonas* populations at both sampling times, except *Pseudomonas* populations in August bulk soil, where there were no significant effects of crop and treatment (Table 3.3). In May, the highest number of total bacteria was found (6.4 log cfu g^{-1} soil) in the bulk soil under wheat rotation and canola rotation, followed by wheat monoculture (5.4 log cfu g^{-1} soil), while canola monoculture supported the lowest number of total bacteria (4.3 log cfu g^{-1} soil). A similar trend was found with the *Pseudomonas* population, where wheat rotation supported highest number of pseudomonas (5.6 log cfu g^{-1} soil), followed by canola rotation (4.8 log cfu g^{-1} soil), wheat monoculture (3.4 log cfu g^{-1} soil), and the lowest number was observed for the canola monoculture (3.0 log cfu g^{-1} soil).

In August, *Pseudomonas* populations in bulk soil were comparable under different monoculture and rotation treatments, while the canola monoculture harbored the lowest number of culturable bacteria, compared to other cropping systems. However, both total bacteria population and *Pseudomonas* population in rhizosphere soil showed fluctuation under different cropping systems. Canola rotation and wheat rotation had the highest total bacteria population (7.6 log cfu g^{-1} soil), followed by canola monoculture (6.9 log cfu g^{-1} soil), and wheat monoculture (6.4 log cfu g^{-1} soil). For the *Pseudomonas* population in rhizosphere soil, wheat rotation had the highest number (6.6 log cfu g^{-1} soil). The total bacteria population and the Pseudomonas population in bulk and rhizosphere soil were higher under ZT for both sampling times, compared to conventional tillage.

At Glenlea Long Term Rotation and Management Research Station field trials, populations of *Pseudomonas* and total bacteria were affected by rotation and management to varying degrees (Table 3.4). The *Pseudomonas* population in bulk soil was stable under different cropping systems at both sampling times. However, organic management supported a higher population of *Pseudomonas* in rhizosphere soil in June, compared to conventional management. The total bacteria population showed relatively higher numbers under organic management, except samples in bulk soil in June, where the grain-only conventional system supported the highest bacterial number (6.1 logcfu g^{-1} soil), compared to other cropping systems.

3.4.2 Evaluation of antagonistic activity of bacterial isolates towards *Sclerotinia sclerotiorum in vitro*

In total, 700 bacterial isolates originating from 10 different treatments (CM, CR, WM, WR, ZT, CT, GO, GC, FO, and FC, 70 isolates per treatment) were screened in a

vitro assay for their ability to suppress *S. sclerotiorum*. Approximately 10% (69 of 700) of all isolates demonstrated antagonistic activity towards *Sclerotinia sclerotiorum* in PDA media (Table 3.5). Of 69 antagonistic isolates, 30 came from Ian Morrison Station, and other 39 isolates were obtained from the Glenlea Long Term Rotation and Management Research Station field trials. Three isolates from CT and 9 isolates from ZT showed antagonistic activity against *S. sclerotiorum*. Twenty-six isolates were obtained from organic farming systems, while 13 isolates came from conventional farming systems.

Sixty-nine isolates were screened for the presence of the *prnD*, *phlD, phzC* and *phzD*, and *pltC* genes by using PCR analysis (Table 3.1 and 3.5). Nineteen out of 69 isolates yielded a 786bp of *prnD* specific amplification product, which is involved in the biosynthesis of the antibiotic pyrrolnitrin (Table 3.5 and Figure 3.3), and 25 isolates had 1150bp amplicons of the *phzC* and *phzD* genes, which is involved in the biosynthesis of phenazine (Table 3.5 and Figure 3.5). When the *phlD* and *pltC* genes were tested, which are involved in the biosynthesis of 2,4 –DAPG and pyoluteorin, it was found that no isolate harbored these two genes.

3.4.3 Bacterial Identification

In total, 25 bacteria demonstrating antagonistic activity towards *S. Sclerotiorum* were identified using the Microlog^{TM} machine. They were identified to the species level (Table 3.5).

3.4.4 HPLC analysis of the antifungal metabolites present in the cell-surface extracts of bacterial isolates

The bacterial cell surface extractions of strain DF306, DF412 were subjected to HPLC analysis for the detection of pyrrolnitrin, phenazine and 2,4-DAPG. The retention

time of strain DF306 showed at 16-18 mins, which was the pyrrolnitrin peak observed in the pure pyrrolnitrin standard sample and positive control strain *P. fluorescens* Pf-5. The amount of pyrrolnitrin produced by DF306 (0.032 AU) was two fold more than what produced by *P. fluorescens* Pf-5 (0.016 AU) (Fig. 3.4). DF412 showed the phenazine peak at retention time 12-14 mins, which was observed in the pure phenazine standard sample and positive control strain PA23. The amount of phenazine produced by DF412 (0.86 AU) was two fold as much as that produced by PA23 (0.4 AU) (Fig. 3.6)

3.4.5 Bacterial richness, diversity and similarity indices based on TRFLP incidence profiles

At the Ian Morrison Research Station, species richness and diversity were highest in the bulk soil of canola rotation; followed by wheat rotation, wheat monoculture, and they were lowest under canola monoculture. However, when species richness and diversity in rhizosphere soil was compared, wheat rotation had the highest number with 105.8 and 3.2, respectively. Zero tillage had similar species richness and diversity both in bulk soil and rhizosphere soil, compared to conventional tillage (Table 3.6). When canola rotation, canola monoculture, wheat rotation and wheat monoculture were compared, hierarchical clustering of TRFs digested with *HallI* showed three distinct clusters including canola monoculture bulk soil (cluster 1), canola rotation bulk, rhizosphere soil and canola monoculture rhizosphere (cluster 2), and both bulk and rhizosphere soil of wheat monoculture and wheat rotation (cluster 3) (Figure 3.7). When zero tillage was compared to conventional tillage, TRFs profiles clustered by bulk and rhizosphere soil type (Figure 3.8).

At the Glenlea Long Term Rotation and Management Research Station, in general, species richness and diversity were affected by the interaction of rotation and management, and the diversity was also affected by management factor. Forage-grain organic had the

highest diversity indices and relatively higher richness, compared to other rotation and management (Table 3.7) systems. Hierarchical clustering of TRFs showed two distinct statistically significant rhizosphere and bulk soil clusters. Within each cluster, the samples were sub-clustered by treatments (organic or conventional) (Figure 3.9).

Table 3.1 List of primers used for PCR analysis in detection of genes involved antibiotic synthesis

Antibiotic	Primer	Primer Sequence (5'-3')	Product Length	Target Gene	Reference Strain	Reference
Phenazine	PCA2a PCA3b	TTGCCAAGCCTCGCTCCAAC CCGCGTTGTTCCTCGTTCAT	1150bp	*phzC* and *phzD*	*P. fluorescens* 2-79	Boronin et al.1995
Pyrrolnitrin	PRND1 PRND2	GGGGCGGGCCGTGGTGATGGA YCCCGCSGCCTGYCTGGTCTG	786bp	*prnD*	*P. fluorescens* Pf5	De Souza and Raaijmakers 2003
2,4-Diacetylphloroglucinol	Phl2a Phl2b	GAGGACGTCGAAGACCACCA ACCGCAGCATCGTGTATGAG	745bp	*phlD*	*P. fluorescens* Pf5	Raaijmaker et al.1997
Pyoluteorin	PLTC1 PLTC2	AACAGATCGCCCCGGTACAGAACG AGGCCCGGACACTCAAGAAACTCG	438bp	*pltC*	*P. fluorescens* Pf5	De Souza and Raaijmakers 2003

Table 3.2 List of PCR Programs used for the detection of antibiotic biosynthetic genes in bacteria antagonistic to *Sclerotinia sclerotiorum*

Antibiotic	Initial Denaturation	Denaturation	Annealing	Extension	Final extension	#Cycles	Source
Phenazine	94°C, 2 min	94°C, 60s	67°C, 45s	72°C, 60s	72°C, 10 min	30	Boronin et al. 1995
Pyrrolnitrin	95°C, 2 min	95°C, 60s	68°C, 60s	72°C, 60s	72°C, 10 min	30	de Souza and Raaijmakers, 2003
2,4-Diacetylphloroglucinol	94°C, 2 min	94°C, 60s	67°C, 45s	72°C, 60s	72°C, 10 min	30	Raaijmakers et al. 1997
Pyoluteorin	95°C, 2 min	95°C, 2 min	67°C, 60s	72°C, 60s	72°C, 10 min	30	de Souza and Raaijmakers, 2003

Table 3.3 Total bacterial population and *Pseudomonas* populations (log cfu g^{-1} soil) at the Ian Morrison Research Station field

Crop	Treatment	May (before planting time) Bulk soil		August (before harvest time) Bulk soil		Rhizosphere soil	
		Total bacteria	*Pseudomonas*	Total bacteria	*Pseudomonas*	Total bacteria	*Pseudomonas*
Canola	Monoculture	4.3[c]	3.0[c]	4.5[b]	3.5	6.9[b]	6.0[b]
Canola	Rotation	6.4[a]	4.8[b]	5.2[a]	3.7	7.6[a]	5.7[b]
Wheat	Monoculture	5.4[b]	3.4[c]	5.1[a]	4.3	6.4[c]	5.9[b]
Wheat	Rotation	6.4[a]	5.6[a]	5.2[a]	3.5	7.6[a]	6.6[a]
SEM		0.03	0.13	0.12	0.16	0.07	0.11
Crop (*P*-value)		<0.0001	<0.0001	0.04	0.12	0.02	0.009
Treatment (*P*-value)		<0.0001	0.004	0.03	0.17	<0.0001	0.06
Crop x Treatment (*P*-		<0.0001	0.29	0.05	0.04	0.01	0.001
Zero tillage		5.2	3.6	5.3	3.7	7.1	6.4
Conventional tillage		4.3	3.0	4.5	3.5	6.9	6.0
SEM		0.03	0.05	0.03	0.05	0.04	0.04
P-value		0.002	0.01	0.0001	0.05	0.06	0.02

[a,b,c] Means with different letters are significantly different for treatments at P<0.05

Table 3.4 Total bacterial population and *Pseudomonas* populations (log cfu g^{-1} soil) at the Glenlea Long Term Rotation and Management Research Station field trials

Rotation	Management	June				August			
		Bulk soil		Rhizosphere soil		Bulk soil		Rhizosphere soil	
		Pseudomonas	Total bacteria	*Pseudomonas*	Total bacteria	*Pseudomonas*	Total bacteria	*Pseudomonas*	Total bacteria
Grain-only	Organic	4.5	5.8[a,b]	5.5[a]	6.4	4.1	6.3[a]	5.2	6.6[b]
	Conventional	4.4	6.1[a]	4.8[a,b]	5.8	4.5	6.0[a,b]	5.3	6.4[b]
Forage-grain	Organic	4.2	5.9[a,b]	5.3[a,b]	5.9	4.8	6.4[a]	5.3	7.5[a]
	Conventional	4.5	5.5[b]	4.5[b]	5.8	4.2	5.6[b]	5.1	6.9[b]
SEM		0.14	0.13	0.18	0.18	0.24	0.13	0.16	0.21
Rotation (*P*-value)		0.66	0.08	0.17	0.17	0.34	0.32	0.51	<0.0001
Management (*P*-value)		0.71	0.73	0.002	0.07	0.85	0.002	0.88	0.008
Rotation x Management (*P*-value)		0.24	0.004	0.97	0.15	0.06	0.10	0.14	0.06

[a,b,c] Means with different letters are significantly different for treatments at P<0.05

Table 3.5 List of bacterial isolates from different cropping systems for the presence of antibiotic biosynthetic genes using specific primer-based PCR.

Isolates	Origin	Identification	Inhibition of mycelia (%)	Number of sclerotia	Pyrrolnitrin	Phenazine
Can10-05 (ck)		*Sclerotinia sclerotiorum* (Fungus pathogen)	0%	25		
Wheat Rotation (WR) vs. Wheat Monoculture (WM)						
DF302	Wheat bulk soil (WR)	*Burkholderia pyrrocinia*	70.0%	15	*	
DF304	Wheat bulk soil (WR)		55.6%	3	*	
DF305	Wheat bulk soil (WR)	*Pseudomonas marginalis*	33.3%	0		
DF312	Wheat rhizosphere (WR)	*P. syringae*				
DF323	Wheat rhizosphere (WR)		40.0%	0		
DF332	Wheat rhizosphere (WR)					
DF359	Wheat rhizosphere (WM)					
DF362	Wheat rhizosphere (WM)					
DF388	Wheat bulk soil (WM)		48.6%	0		
Canola Rotation (CR)						
DF301	Canola bulk soil	*P. fluorescens* biotype F	66.7%	4		
DF456	Canola rhizosphere		45.7%	0		
DF473	Canola rhizosphere		33.3%	0		

DF492	Canola rhizosphere		45.7%	0		*
DF495	Canola rhizosphere		40.0%	0		*
Zero tillage (ZT) vs. Conventional tillage (CT) (Canola monoculture)						
DF405	Canola rhizosphere (ZT)	*P. fluorescens* biotype A	58.2%	8	*	*
DF412	Canola rhizosphere (CT)	*P. fluorescens*	66.0%	10	*	*
DF415	Canola rhizosphere (CT)	*P. fluorescens* biotype C	51%	10		
DF417	Canola rhizosphere (CT)		42.7%	0		
DF423	Canola bulk soil (ZT)	*P. fluorescens* biotype C	82.0%	10	*	
DF425	Canola bulk soil (ZT)		54.3%	0		*
DF426	Canola bulk soil (ZT)		62.7%	0		*
DF433	Canola bulk soil (ZT)	*P. fluorescens* biotype G	63.8%	8	*	
DF441	Canola rhizosphere (ZT)		42.7%	0		*
DF442	Canola rhizosphere (ZT)		40%	0	*	
DF444	Canola rhizosphere (ZT)	*P. fluorescens*	60.2%	5		*
DF448	Canola rhizosphere (ZT)	*Dermacoccus nishinomiyaesis*	40.1%	15	*	*
DF453	Canola rhizosphere (ZT)		48.5%	0	*	
Forage-grain Organic (FO) vs. Forage-grain Conventional (FC)						
DF306	Flax rhizosphere (FC)	*P. marginalis*	100%	0	*	
DF440	Wheat bulk soil (FO)		100%	0	*	
DF512	Flax rhizosphere (FO)		46.7%	8		*

DF515	Flax rhizosphere (FO)	*P. marginalis*	50.0%	10	*	*
DF516	Wheat Bulk soil (FC)	*P. synxantha*	47.2%	11		*
DF517	Wheat bulk soil (FC)		43.3%	5		
DF525	Wheat bulk soil (FO)		42.7%	0		*
DF526	Wheat bulk soil (FO)		66.7%	11	*	
DF527	Wheat bulk soil (FO)		50.0%	10		
DF530	Wheat rhizosphere (FO)		62.2%	0	*	*
DF532	Wheat rhizosphere (FO)		65.8%	0	*	*
DF534	Wheat rhizosphere (FO)		66.7%	2		
DF535	Flax rhizosphere (FO)	*P. fluorescens*	66.7%	11	*	*
DF537	Flax rhizosphere (FO)		62.7%	0		*
DF539	Flax rhizosphere (FO)	*P. marginalis*	43.3%	6	*	*
DF543	Wheat rhizosphere (FO)		60.0%	0		
DF544	Wheat rhizosphere (FO)					
DF547	Wheat rhizosphere (FO)		60.0%	0		
DF551	Flax bulk soil (FC)		60.0%	0		*
DF552	Flax bulk soil (FC)		57.1%	0		
DF557	Flax bulk soil (FC)		46.2%	0		*
DF584	Flax rhizosphere (FC)		42.7%	5		
DF593	Flax rhizosphere (FC)		42.7%	0		*

Grain_only Organic (GO) vs. Grain_only Conventional (GC)

DF327	Flax rhizosphere (GC)		33.3%	0		*
DF382	Flax rhizosphere (GC)		70.0%	0	*	*
DF501	Flax rhizosphere (GC)	*P. synxantha*	40.3%	9		*
DF602	Flax rhizosphere (GO)		36.2%	0		*
DF603	Flax rhizosphere (GO)		50.0%	0		*
DF606	Flax rhizosphere (GO)		50.0%	5		
DF607	Flax rhizosphere (GO)	*Bacillus cereus*	50.0%	0		
DF614	Flax rhizosphere (GO)		40.0%	8	*	
DF615	Wheat rhizosphere (GO)	*P. asplenii*	66.6%	0		*
DF617	Wheat rhizosphere (GO)	*P. fluorescens* biotype A	33.3%	8		
DF618	Wheat rhizosphere (GO)	*P. fluorescens* biotype G	33.3%	9		
DF619	Wheat rhizosphere (GO)	*P. fluorescens* biotype G				
DF620	Wheat rhizosphere (GC)	*P. synxantha*	66.6%	0		
DF622	Wheat rhizosphere (GC)	*P. marginalis*	33.3%	0		
DF623	Wheat rhizosphere (GC)	*P. marginalis*	33.3%	0		
DF625	Bulk soil (GO)		66.6%	5		
DF626	Bulk soil (GO)		60%	10		
DF630	Bulk soil (GC)		46.2%	5		
DF642	Bulk soil (GC)		62.7%	2		

Table 3.6 Richness and Diversity indices calculated from TRFLP incidence profiles of bulk and rhizosphere soil at the Ian Morrison Research Station field trials

Crop	Treatment	May (before planting time)		August (before harvest time)			
		Bulk soil		Bulk soil		Rhizosphere soil	
		Richness[1]	Diversity[2]	Richness[1]	Diversity[2]	Richness[1]	Diversity[2]
Canola	Monoculture	18.1	2.3[b]	14.6[b]	2.2	38.6[b]	2.3[b]
Canola	Rotation	40.4	3.4[a]	31.2[a]	2.4	79.5[ab]	2.8[ab]
Wheat	Monoculture	16.8	2.5[b]	18.5[b]	2.2	47.8[b]	2.4[b]
Wheat	Rotation	24.0	2.7[b]	22[ab]	2.3	105.8[a]	3.2[a]
SEM		9.2	0.3	6.7	0.1	21.1	0.28
Crop (*P*-value)		0.2	0.01	0.03	0.1	0.01	0.01
Treatment (*P*-value)		0.09	0.09	0.4	0.6	0.32	0.21
Crop x Treatment (*P*-		0.3	0.02	0.09	0.3	0.1	0.2
Zero tillage		20.1	2.6	17.2	2.7	38.6	2.4
Conventional tillage		18.5	2.3	15.6	2.2	30.0	2.3
SEM		11.9	0.26	11.5	0.13	5.64	0.2
P-value		0.54	0.41	0.27	0.07	0.34	0.82

[a,b,c] Means with different letters are significantly different for treatments at P<0.05.

[1]Based on Chao1-richness estimator.

[2]Based on Shannon diversity estimator.

Table 3.7 Richness and Diversity indices calculated from TRFLP incidence profiles at the Glenlea Long Term Rotation and Management Research Station field trials

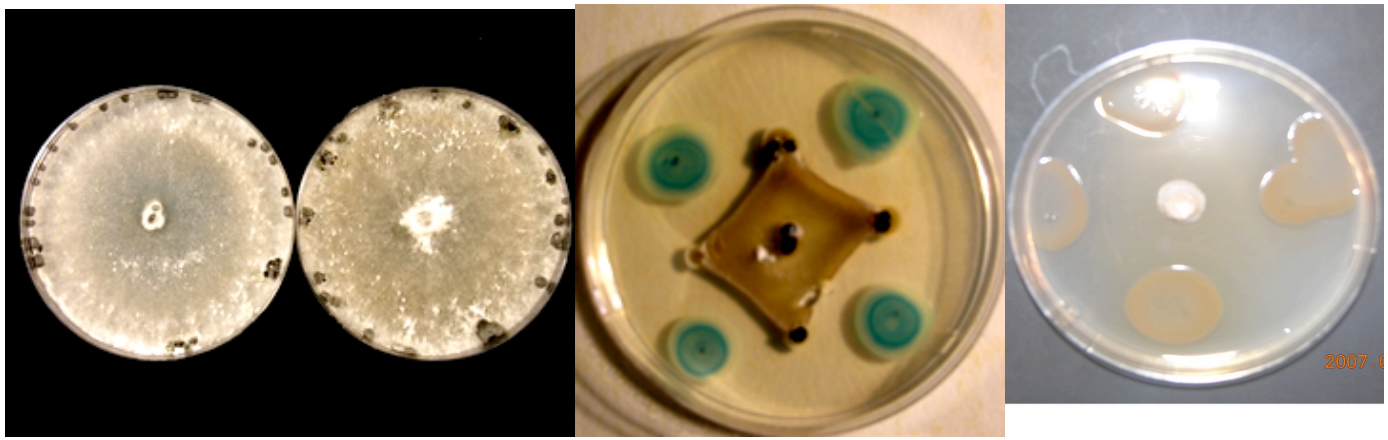
Rotation	Management	June				August			
		Bulk soil		Rhizosphere soil		Bulk soil		Rhizosphere soil	
		Richness[1]	Diversity[2]	Richness[1]	Diversity[2]	Richness[1]	Diversity[2]	Richness[1]	Diversity[2]
Grain-only	Organic	43.3	2.8^{bc}	84.0	3.1^{ab}	63.3	2.8^{bc}	17.9^{c}	2.0^{c}
	Conventional	69.7	3.0^{b}	111.5	2.9^{b}	56.4	2.7^{c}	68.4^{b}	2.8^{b}
Forage-grain	Organic	83.4	3.3^{a}	173.7	3.2^{a}	74.3	3.7^{a}	100.2^{a}	3.5^{a}
	Conventional	60	2.6^{c}	76.5	2.4^{c}	52.6	3.0^{b}	13.3^{c}	1.6^{c}
SEM		10.1	0.17	23.3	0.22	12.0	0.15	12.3	0.16
Rotation (*P*-value)		0.17	0.21	0.21	0.008	0.75	<0.0001	0.26	0.05
Management (*P*-value)		0.89	0.005	0.13	<0.0001	0.23	0.0002	0.15	0.0004
Rotation x Management (*P*-value)		0.04	0.0003	0.02	0.002	0.52	0.0009	0.0008	<0.0001

[a,b,c] Means with different letters are significantly different for management at P<0.05.

[1]Based on Chao1-richness estimator.

[2]Based on Shannon diversity estimator.

Figure 3.1 Soil sampling in the canola monoculture field at the Ian N. Morrison Research Station in the year 2006.



Control **DF301** **DF306**

Figure 3.2 Dual plate assays on Typticase soy agar/ Potato dextrose agar (TSA/PDA) to test the ability of bacterial isolates to suppress the mycelial growth and sclerotia formation of *S. sclerotiorum*. Note mycelial growth and sclerotia formation of control (*S. sclerotiorum* without challenging with bacteria) covered the entire agar surface. *P. fluorescens* biotype F strain DF301 had 66.7% inhibition of mycelial growth and significantly reduced the formation of sclerotia of *S. sclerotiorum. Pseudomonas marginalis* strain DF306 had 100% inhibition of mycelial growth and sclerotia formation of *S. sclerotiorum*.

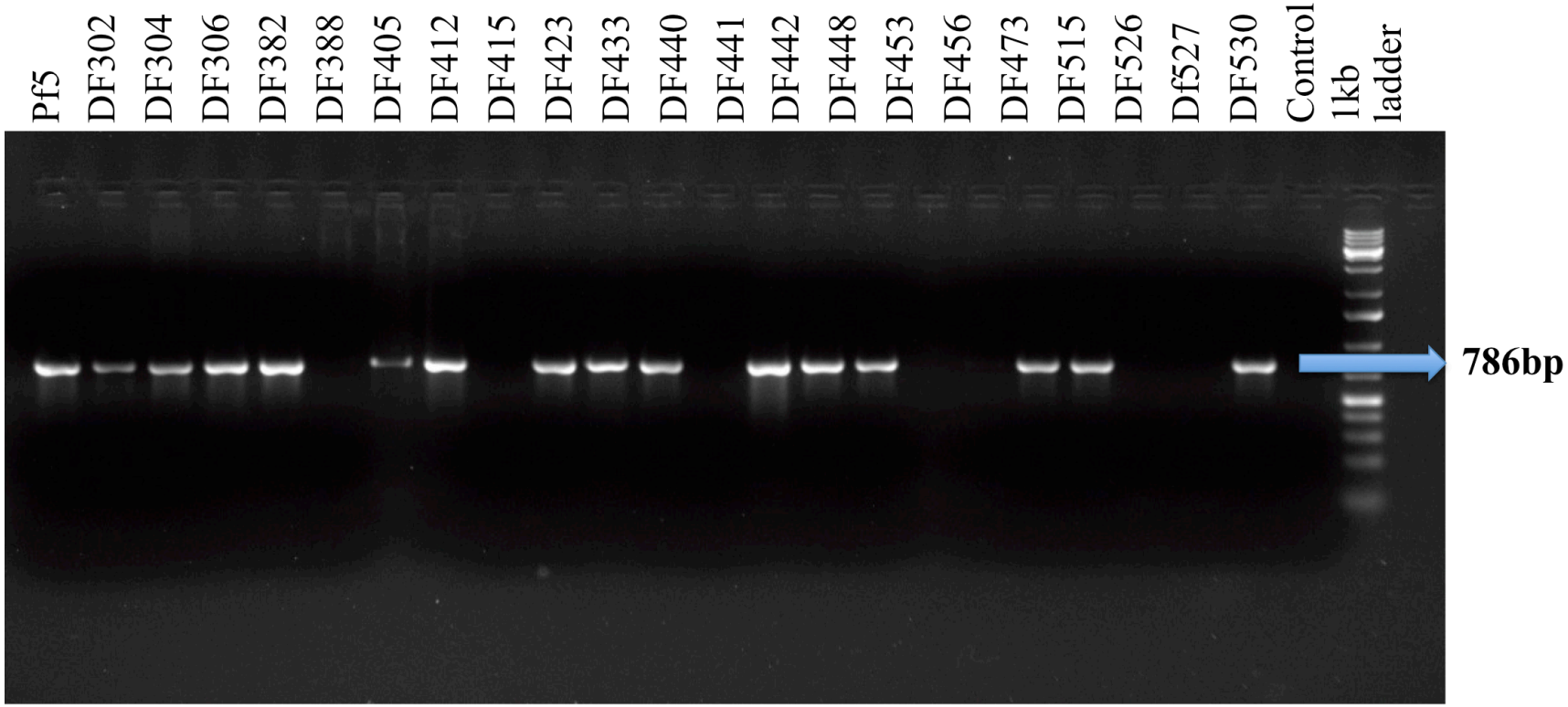


Figure 3.3 Screening for the presence **of** *prnD* gene with specific primers prnD1 and prnD2 (positive control strain: Pf5) for pyrrolnitrin. Isolates DF302, DF304, DF306, DF382, DF405, DF412, DF423, DF433, DF440, DF442, DF448, DF453, DF473, DF515, and DF530 produced the 786bp band indicative of the presence of the pyrrolnitrin biosynthetic genes.

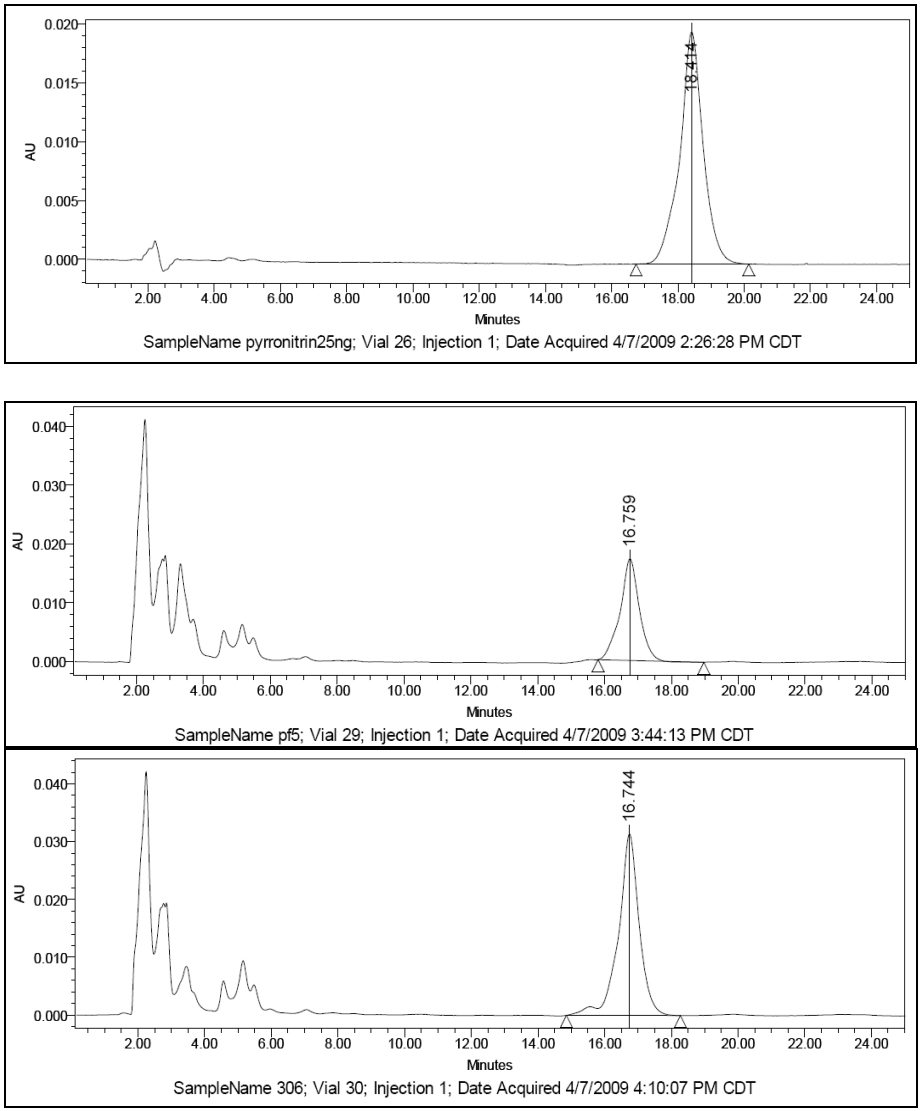


Figure 3.4 HPLC analysis of bacterial broth culture extracts for the presence of pyrrolnitrin. A pyrrolnitrin peak was observed in the range of retention time of 16-18 minutes. Upper picture: pure pyrrolnitrin standard sample; middle picture: *Pseudomonas fluorescens* Pf-5 (positive control); bottom picture: *Pseudomonas marginalis* strain DF306

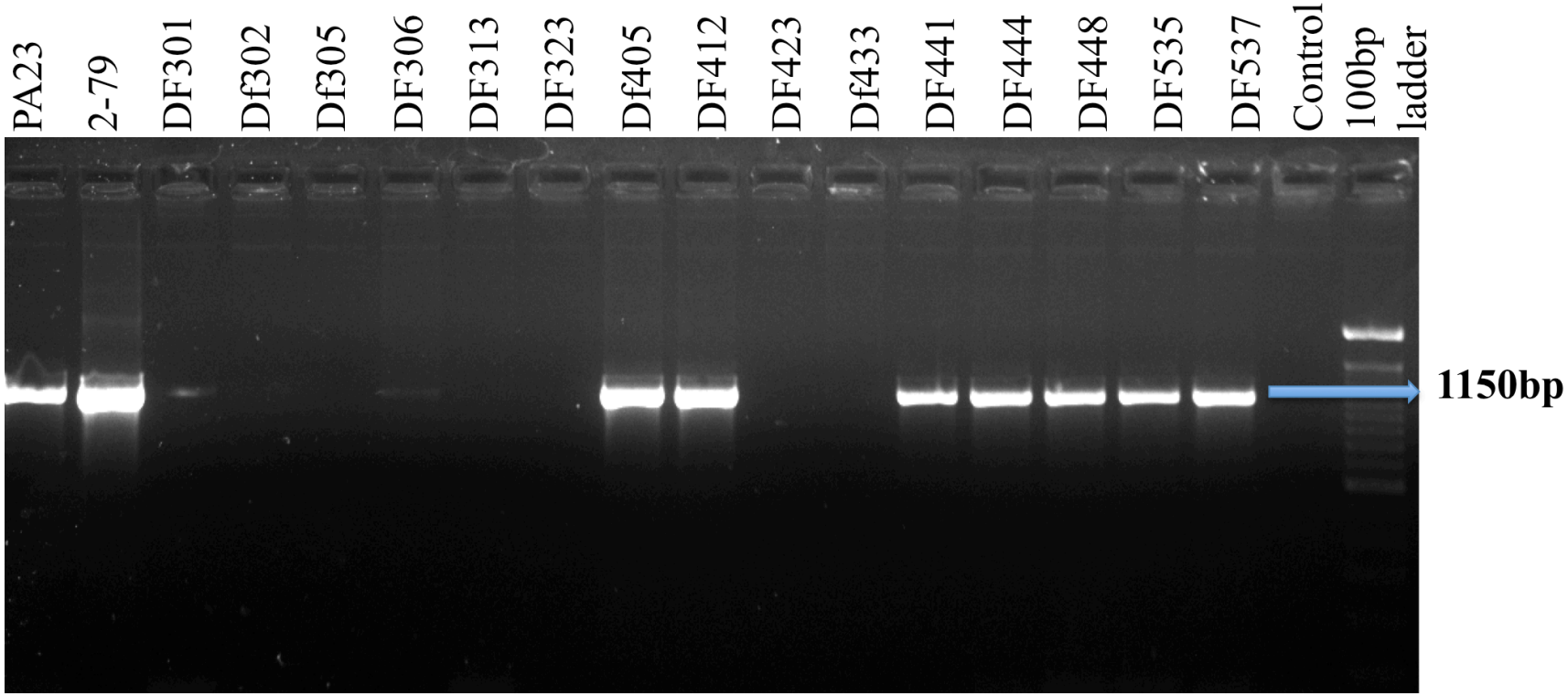


Figure 3.5 Screening for the presence of phzC and phzD genes with specific primers PCA2a and PCA3b (Positive control strain *P. fluorescens* 2-79 and *P. chlororaphis* PA23) for phenazine. Isolates DF405, DF412, DF441, DF444, DF448, DF535, and DF537 produced 1150bp band indicative of the presence of phenazine biosynthetic genes.

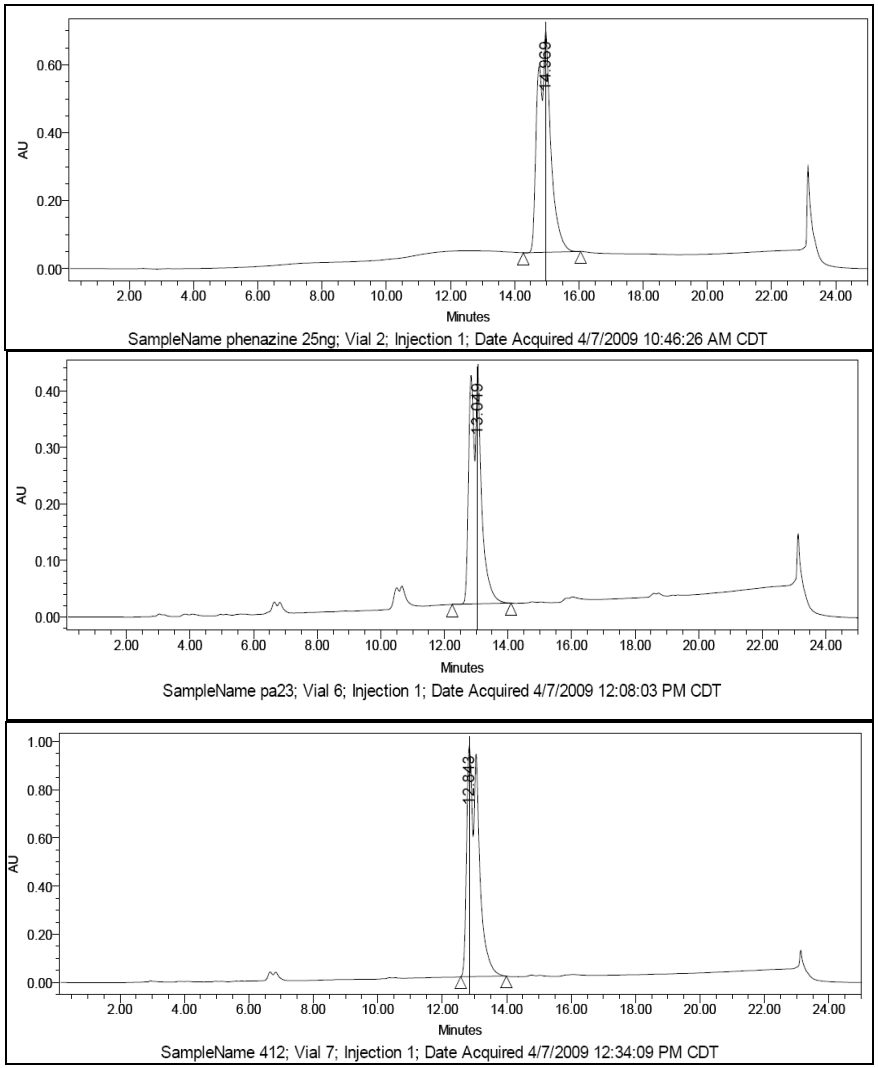


Figure 3.6 HPLC analysis of bacterial broth culture extracts for the presence of Phenazine. Phenazine peaks were observed in the range of retention time of 12-16 minutes. Upper figure: pure phenazine standard sample; middle figure: *Pseudomonas chlororaphis* PA23 (Reference strain); bottom figure: *Pseudomonas fluorescens* strain DF412

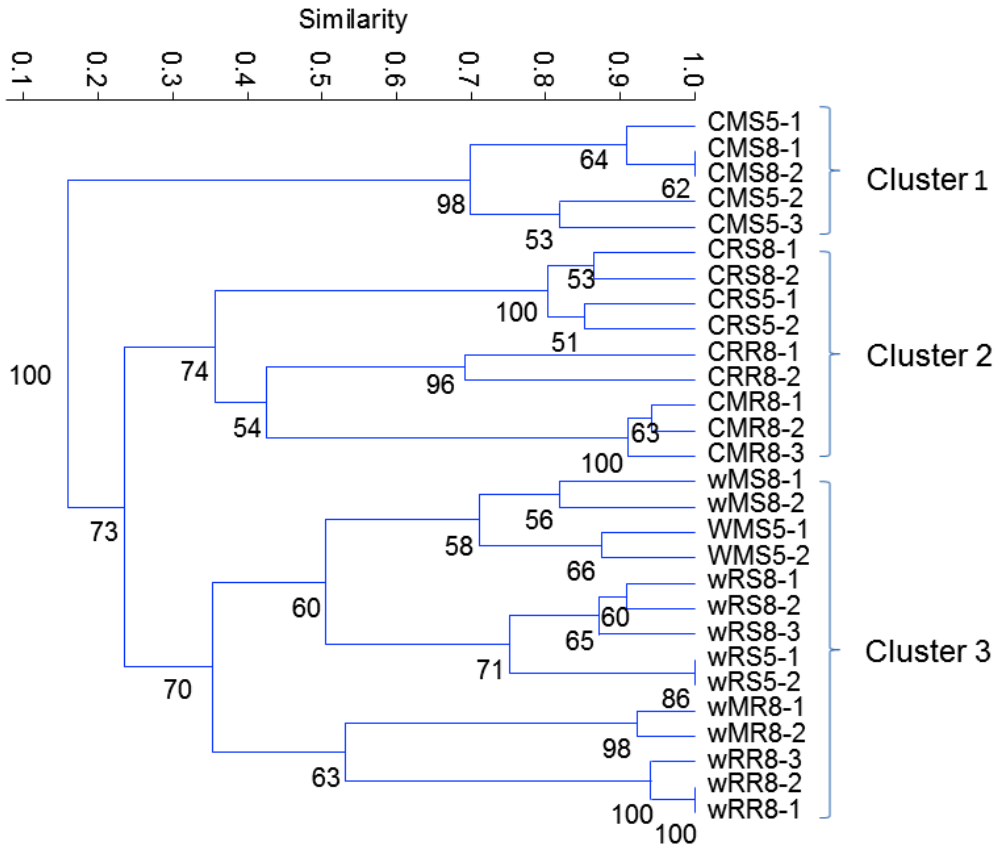


Figure 3.7 UPGMA cluster based on Jaccard index of similarity of bacterial communities between wheat-canola-oat-pea rotation, wheat monoculture, and canola monoculture treatments calculated from T-RFLP incidence profiles of bulk and rhizosphere soil samples amplified using 27f and 342r primers. CMS5 (bulk soil sampled in canola monoculture plot in May, 2006); CMS8 (bulk soil sampled in canola monoculture plot in Aug., 2006); CRS8 (bulk soil sampled in canola rotation plot in Aug., 2006); CMR8 (rhizosphere soil sampled in canola monoculture plot in Aug., 2006); CRR8 (rhizosphere soil sampled in canola rotation plot in Aug., 2006); WMS5 (bulk soil sampled in wheat monoculture plot in May, 2006); WMS8 (bulk soil sampled in wheat monoculture plot in Aug., 2006); WRS5 (bulk soil sampled in wheat rotation plot in May, 2006); WRS8 (bulk soil sampled in wheat rotation plot in Aug., 2006); WMR8 (rhizosphere soil sampled in wheat monoculture in Aug., 2006); WRR8 (rhizosphere soil sampled in wheat rotation plot in Aug., 2006).

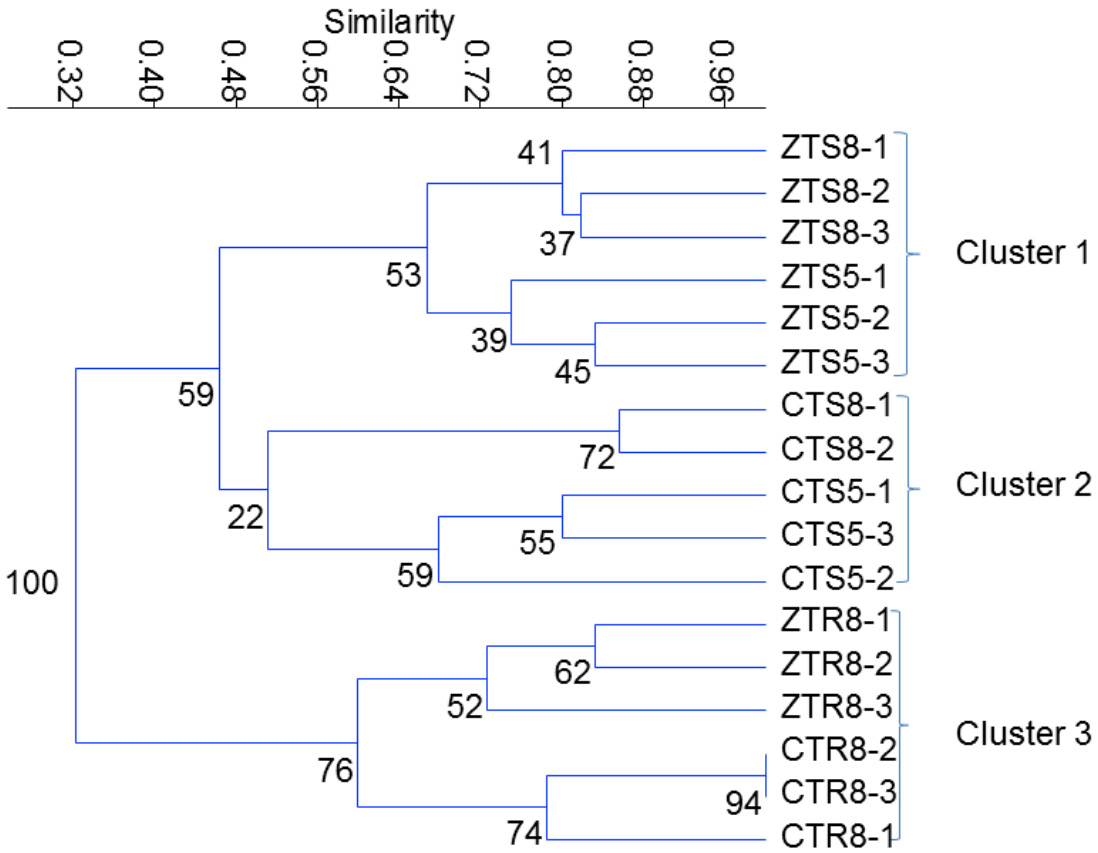


Figure 3.8 UPGMA cluster based on Jaccard index of similarity of bacterial communities between zero tillage and conventional tillage treatments calculated from T-RFLP incidence profiles of bulk and rhizosphere soil samples amplified using 27f and 342r primers. ZTS8 (bulk soil sampled in zero tillage plot in Aug., 2006); ZTS5 (bulk soil sampled in zero tillage plot in May, 2006); CTS8 (bulk soil sampled in conventional tillage plot in Aug., 2006); CTS5 (bulk soil sampled in conventional tillage plot in May, 2006); ZTR8 (rhizosphere soil sampled in zero tillage plot in Aug., 2006); CTR8 (rhizosphere soil sampled in conventional tillage plot in Aug., 2006).

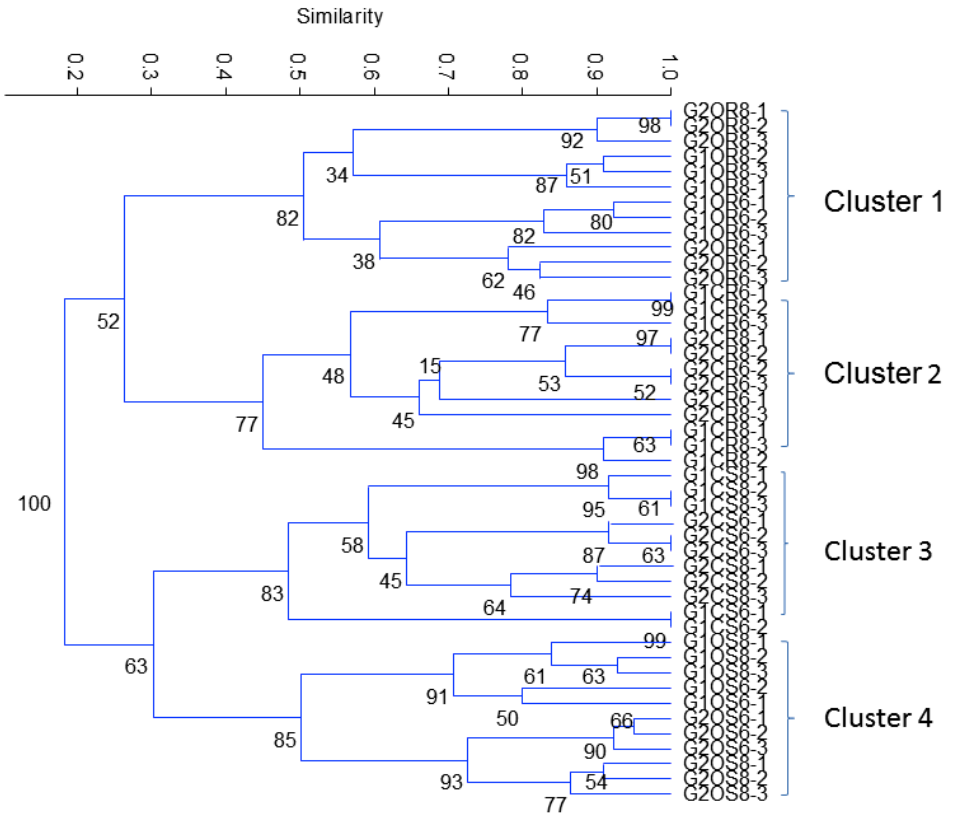


Figure 3.9 UPGMA cluster based on Jaccard index of similarity of bacterial communities between Glenlea rotation and management treatments calculated from T-RFLP incidence profiles of bulk and rhizosphere soil samples amplified using 27f and 342r primers. G2OR8 (rhizosphere soil sampled in Forage_grain organic plot in Aug., 2008); G1OR8 (rhizosphere soil sampled in Grain_only organic plot in Aug., 2008); G1OR6 (rhizosphere soil sampled in Grain_only organic plot in June 2008); G2OR6 (rhizosphere soil sampled in Forage_grain organic plot in June 2008); G2CR8 (rhizosphere soil sampled in Forage_grain conventional plot in Aug 2008); G1CR8 (rhizosphere soil sampled in Grain_only conventional plot in Aug 2008); G2CR6 (rhizosphere soil sampled in Forage_grain conventional plot in June 2008); G1CR6 (rhizosphere soil sampled in Grain_only conventional plot in June 2008); G2CS8 (bulk soil sampled in Forage_grain conventional plot in Aug 2008); G1CS8 (bulk soil sampled in Grain_only conventional plot in Aug 2008); G1OS8 (bulk soil sampled in Grain_only organic plot in Aug 2008); G2OS8 (bulk soil sampled in Forage_grain organic plot in Aug 2008); G2CS6 (bulk soil sampled in Forage_grain conventional plot in June 2008); G1CS6 (bulk soil sampled in Grain_only conventional plot in June 2008); G2OS6 (bulk soil sampled in Forage_grain organic plot in June 2008); G1OS6 (bulk soil sampled in Grain_only organic plot in June 2008).

3.5 Discussion

In this study, we applied both culture dependent and molecular methods to evaluate the influence of different cropping systems on the richness, diversity and function of bacterial populations in soil. One detection system, which was based on different media, assessed the total culturable bacteria and *Pseudomonas* population. A conventional PCR assay was also performed with the 4 primer sets, including PCA2a/PCA3b, PRND1/PRND2, Phl2a/Phl2b, and PLTC1/PLTC2, to test the prevalence of *phzC* and *phzD*, *prnD*, *phlD* and *pltC* genes in pure culture isolates obtained from field plots under different cropping systems. These are important genes encoding the biosynthesis of antibiotics phenazine, pyrrolnitrin, 2,4-diacetylphloroglucinol and pyoluteorin. The second system employed terminal restriction fragment length polymorphism (TRFLP) to evaluate changes in bacterial communities under different cropping systems.

The culture-based methods have shown the differentiation of the total bacterial and *Pseudomonas* populations between the bulk soil and rhizosphere soil (Table 3.3 and 3.4), with higher log CFU found in rhizosphere soil. The effect of rhizosphere on bacterial communities is well documented in the literature. Differences result from root exudates, which supply carbon substrates as energy as well as nutrient sources for bacterial community growth and development, which is lacking in bulk soil.

Compared to time-consuming procedure of random isolation and screening antibiotic producing bacteria from natural environments, specific primer based PCR screening techniques helps to accelerate the process of detection of antibiotic producing bacteria (Mcspadden-Gardener 2007). It was found that potential pyrrolnitrin and phenazine producers were more prevalent than the 2,4-DAPG and pyoluteorin producers in

our field trials. These two antibiotics were found to be more prevalent under the zero tillage and organic farming systems (Table 3.3). Garbeva et al (2004) reported *prnD* gene abundance in the soil samples depended on agricultural practices, with the highest densities of *prnD* found in permanent grassland, while lower or varying densities were present in long-term arable land. It is well accepted that plant type can strongly affect the microbial composition in soil as well the expression of antibiotic production (Smith et al 1999; Raaijmakers et al 2002). Zero tillage and organic farming systems are richer in roots and root exudates than conventional tillage and conventional farming systems; therefore, organisms may be stimulated to produce antibiotics (Ryan et al. 2009; Haas and Défago 2005; Entz et al. 2004).

We could not obtain isolates which produce 2, 4-DAPG and pyoluteorin (Plt). This result was consistent with previous studies done in our lab (Ramarathanam 2007; Zhang, 2004). Most Phl producers were isolated from monoculture soil, especially wheat suppressive soils (Haas and Défago 2005). Therefore, it could be hypothesized that Phl producers could result from long-term monoculture wheat soil. However, our wheat monoculture field was only around 3 years when the soil was sampled. As such, the growing periods might not have been long enough. In addition, it was reported that cultivation of oat had negative effects on the population of Phl producers (Weller et al 2002). Some of our field trials were rotated with oat and this could lead to reduced Phl producers in our field soil. Mcspadden-Gardener and his colleagues (2007) also reported farm management practices could significantly alter the relative abundance of Phl producers by simply altering the soil environment. They compared continuous cropping to rotation cropping systems and found that abundance of 2,4-DAPG producers were

increased under conditions of elevated soilborne disease pressure such as *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp.

In this study, we found the species *P. marginalis* can produce pyrrolnitrin and phenazine (such as isolates DF306, DF515 and DF539). To our knowledge, this is the first report that this *Pseudomonas* species produces pyrrolnitrin and phenazine. *P. marginalis* is an opportunistic pathogen, which can cause soft rot disease on several crops, such as flax, tomato and potato. Further work should be done to test these strains for pathogenicity and potential biocontrol traits.

Collectively, the complexity of ecological interactions can alter the subpopulations of antibiotic producing *Pseudomonas* spp., which include, soil edaphic characteristics, plant hosts, , farming practices and even *Pseudomonas* populations themselves.

The results obtained with the direct molecular analyses (TRFLP) in this study were consistent with the results from culturable media and supported the general view that the agriculture management and crop type influence populations of bacteria in soil to varying degrees. For example, the diversity and richness of bacterial communities are significantly higher in the crop rotation soil than in the monoculture crop soil (Table 3.6). The different crops could release different types and concentrations of root exudates, such as carboxylates, amino acids, sugars, and vitamins. These different "foods" provided by different crops could support a wide range of bacterial communities in the soil. Hierarchical clustering of T-RFs digested by *Hal1* showed distinct statistically significant (MRPP tests: $P<0.02$) rhizosphere soil and bulk soil clusters (Figure3.7, 3.8, and 3.9), demonstrating that the rhizosphere strongly affects bacterial community composition. T-RFs cluster also showed that bacterial communities in bulk soil of canola monoculture had the lowest similarity to other cropping systems (canola rotation, wheat monoculture and rotation)

(Figure 3.7). It was reported that arbuscular mycorrhizal fungi (AMF) could take part in shaping bacterial communities in the soil ecosystem (Toljander et al 2007). It was well known that canola is a completely non-mycorrhizal crop. As such, growing canola continuously could diminish AMF in soil; therefore it would result in differences of bacterial communities, compared to other cropping systems. When canola rotates with other crops, such as wheat, oat and pea, mycorrhizal hyphae left in the soil may help to maintain bacterial communities.

Zero tillage leaves crop residue on the soil surface, which allows the accumulation of saprophytic fungi and gram-negative bacteria in ZT surface soil (Doran et al 1998; Mozafar et al 2000; Wortmann et al 2008). Our results showed the total culturable bacteria and *Pseudomonas* spp. were higher in soil under ZT (Table 3). *Pseudomonas* spp. belongs to gram-negative bacteria. Furthermore, the richness and diversity based on the TRFLP profiles was numerically higher in ZT soil, even though not statistically higher than those in CT soil. The UPGMA cluster (Figure 3.8) also showed bacterial communities in ZT soil were significantly different from those in CT soil. Several studies reported CT could disrupt fungal mycelial networks and opportunistic decomposers due to soil disturbance (Mozafar et al 2000; Drijber et al 2000; Wortmann et al 2008). It has been reported that bacterial communities are influenced by fungal communities too (Toljander et al 2007).

TRFLP showed the richness and diversity of bacterial communities were significantly influenced by management (organic vs. conventional) and interaction of rotation (grain-only vs. forage-grain) and management (Table 3.7). Our results were consistent with other studies (Mader et al 2002 Esperschutz et al 2007), reporting that organic farming systems enhance microbial diversity in soil compared to the conventional systems. Weeds were more diverse in organic farming systems and could lead to higher

richness and diversity of bacterial consortia (Entz et al 2004). UPGMA cluster analysis of the TRFLP profile illustrated the root systems played a major role in shaping bacterial communities, with two main clusters separating bacterial communities in rhizosphere soil from bulk soil. Within the main clusters, organic and conventional farming practices played a role in influencing bacterial communities (Fig. 3.9). Conventional farming systems widely use inorganic fertilizers, and pesticides, which could provide xenobiotic substrates for certain bacteria as "food", therefore boosting their growth. (De Schrijver et al 1999; Lee 2004; Akar et al 2005). In terms of richness and diversity indices calcultated from TRFLP at the Glenlea Long Term Rotation and Management Research Station , the effect of the interaction between rotation and management is significant. Compared to grain-only rotation, forage-grain increase N availablity due to N fixation by alfalfa (Welsh et al 2009). This nitrogen substrates also can influence the diversity and richness of bacterial communities.

The methods we used to assess the richness, diversity and function of bacterial communities had their biases. The majority of bacterial communities cannot be cultured. Closely related bacteria will typically generate a TRF of the same size, but unrelated bacteria can also share a TRF in many cases, which could result in a loss of useful information. Thus, the exact diversity of bacterial communities is underestimated. Furthermore, each bacterial community member or operational taxonomic unit (OTU) is measured by the length of the TRFs of hydrolyzed PCR amplicons. However, in complex communities, there is rarely a one-to-one correspondence between a T-RF length and a specific member of the microbial community (Thies 2007). As such, phylogenetic results based on T-RF length, not on sequence information, should be interpreted with caution.

Neither culturable methods nor TRFLP methods can achieve a sufficient sampling depth to assess whole bacterial communities in soil. Higher resolution methods as they become available, such as pyrosequencing or metagenomics shotgun sequencing must be applied in order to gain a comprehensive profile of bacterial consortia under different cropping systems.

4.0 THE INFLUENCE OF TILLAGE AND CROP ROTATION ON STRUCTURE AND COMPOSITION OF BACTERIAL COMMUNITIES

4.1 Abstract

High-throughput pyrosequencing of V1 to V3 regions of bacterial 16S rRNA genes was used to investigate the effect of crop rotation and tillage on the bacterial communities. The test trials were set at the Ian Morrison Research Station, Carman, Manitoba, Canada. Randomized complete block design was conducted on the monoculture (canola and wheat) and rotation treatments (wheat-canola-oat-pea rotation). In addition, two tillage treatments (conventional and zero tillage) were set on canola monoculture plots. Bacterial community composition was compared among wheat and canola monocultures, wheat and canola rotations, and conventional and zero tillage's. A total of 14 phyla and 172 genera were identified. The results revealed that crop was the main discriminator for bacterial community structure; however, farming practices, such as rotation, monoculture or tillage, also affected the community composition to varying degrees. The composition of bacterial community under canola was significantly different from that under wheat, for which class of Deltaproteobacteria and genera of *Blastococcus*, *Arthrobacter*, *Pseudonocardia*, *Solirubrobacter*, *Brevundimonas*, *Pseudolabrys* showed significant variation. Furthermore, the bacterial communities under canola monoculture also showed shifting, compared to canola rotation, whereas the bacterial communities under wheat monoculture and wheat rotation were more similar. Compared to canola monoculture, canola rotation supported relatively higher abundance of Proteobacteria phyla and class of Alphaproteobacteria, and less abundance of Actinobacteria, Gemmatinomadetes and Chloroflexi. It was also found that bacterial communities did not significantly shift by different tillage systems. Integrating canola into cropping systems could increase some bacterial populations, such as *Pseudomonas, Stenotrophomonas*, and *Rhizobium*. These bacterial populations could help crops against pathogens dwelling in the soil. To the best of our knowledge, this is the first

report to compare the change in bacterial community structure under canola monoculture and rotation with wheat by using high throughput technology 454-pyrosequencing.

4.2 Introduction

It is well known that soil-dwelling microorganisms can be of benefit to the quality of soil, which is crucial to maintain health and productivity of crops. Soil-dwelling microorganisms play a central role in innumerable processes and functions carried out in soils, such as organic residues decomposition, nutrient cycling, soil aggregation formation, xenobiotic degradation, and Nitrogen fixation. Moreover, microorganisms can directly benefit crop production via versatile mechanisms including suppression of plant pathogens, induction of resistance in the plant host, and direct stimulation of plant growth (Kloepper et al 2004, Haas and Défago 2005).

Previous studies have demonstrated that different agriculture practices affect the diversity and function of soil microorganisms (Mader et al 2002, Esperschutz et al 2007, Sugiyama et al 2010). Tillage is a common practice in modern agriculture that involves mechanical manipulation of soil to enhance decomposition of crop residues, and to prepare seedbeds for planting. It also serves as a method of post-emergence weed control and a management strategy to reduce the incidence of diseases and pests. However, extensive tillage leads to soil erosion and environmental pollution. There are different types of tillage systems: conventional tillage (CT) and zero tillage (ZT) in which the crop is planted directly into the previous crop's stubble with minimal soil disturbance. It was found that ZT could reduce soil erosion and increase soil organic matter and microbial biomass compared to CT (Drijber et al 2000, Kabir 2005). However, a drawback of ZT is that some soilborne plant diseases can reach damaging levels as pathogens survive on crop residues left on the

soil surface (Guo et al 2005). An appropriate agricultural strategy, such as crop rotation can prevent this problem by excluding pathogen hosts, especially for pathogens with limited host range.

Crop monoculture and rotation are two opposite agriculture practices. Monoculture is defined as the practice of growing the same crop in the same soil year after year, whereas rotation implied temporal sequences in which different crops are grown on the same land (Las 2003). Many studies comparing monoculture to rotation concerned with economic and pathological aspects (Shipton 1977, Zentner et al 1995) have yielded contradictory results. Some studies showed that rotation increased yield and reduced the incidence of disease owing to eradicating the pathogen host and increasing niche heterogeneity of the rhizosphere (Govaerts et al 2007). Whereas others showed that with proper approaches, such as using resistant cultivars and proper fertilizers or management of volunteer wheat, monoculture practices might maintain similar yields compared to rotational practices for a short period of time (Cook and Weller, 2004). However, the effect on soil bacterial communities has not been studied extensively, especially in Canada.

Canola is the number one cash crop in Canada, contributing $15.4 billion to the Canadian economy (Canola Council of Canada, 2012). Wheat is another important crop in Canada, with annual export revenue of $5.4 billion. Owing to high price per bushel, growers in Canada are trying to grow canola in monoculture. Wheat used to grown in monoculture in the Canadian prairies but recently some farmers have adapted canola-wheat rotations with other crops. Within the Canadian Prairies, over 50% of seeded acres are run under ZT and most agricultural land is managed under some form of reduced tillage (70%) to decrease soil disturbance (Statistics Canada, 2008).

In this manuscript we assessed the bacterial community shifts under different

agricultural practices (monoculture vs. rotation, ZT vs. CT) in Manitoba, Canada, using high-resolution pyrosequencing methodology.

4.3 Materials and Methods

4.3.1 Experimental design, and soil sample collection

The experiments were conducted at the Ian Morrison Research Station located in Carman Manitoba, Canada. Canola monoculture (CM) trial, wheat monoculture trial (WM), wheat-canola-oat-pea rotation (CR or WR) trial as well as ZT and CT trials took place in 2003. The soil type was a Regnold loam. A randomized complete block design with three replicates was used for the monoculture and rotation trials. Conventional and zero tillage practices were conducted on the canola monoculture trial. Conventional tillage was carried out using a deep tiller with tine harrow after canola was harvested in the fall of 2005 and using a cultivator with tine harrow and coil packer in the following spring of 2006.

The bulk soils were randomly collected in late May 2006 (before seeding) and August 2006 in wheat and canola plots. Bulk soils were sieved (2mm) to remove crop residues and stones. Each sample was mixed well in plastic bags and kept at -20 °C until DNA was extracted. Bulk soil DNA under the same treatment was pooled and prepared for pyrosequencing.

4.3.2 DNA extraction from soils

Before soil DNA extraction, the pre-washed step was introduced to remove PCR inhibitors including humic acid, covalent cations and other easily dissolved organic compounds as described by He et al (2005). The total soil DNA was then extracted from the pre-washed soil samples using the PowerSoil DNA isolate kit according to the

manufacturer's specifications (Mobio Labs, Solana Beach, CA). The DNA quality was tested by amplifying variable regions of V1-V2 of the 16S rDNA genes using forward primer 27F (AGAGTTTGATCMTGGCTCAG) and reverse primer 342R (CTGCTGCSYCCCGTAG) (Khafipour et al 2009), indicating that the quality of extracted DNA was sufficient for further PCR application. The average DNA yield was 10 ng/ μl.

4.3.3 Pyrosequencing

DNA samples were pyrosequenced using bacterial tag-encoded GS FLX-Titanium amplicons as described by Dowd et al (2008) and Li et al (2012). In brief, a one-step PCR (35 cycles) with primer 27F, which covered the variable regions V1 to V3 of the bacterial 16S rRNA genes, was performed in a mixture of Hot Start, HotStar high fidelity Taq polymerases and Titanium reagents. The pyrosequencing procedures were carried out at the Research and Testing Laboratory (Lubbock, TX; http://www.Researchandtesting.com).

Pyrosequencing raw data were edited, categorically transformed and classified as described by Li et al (2012). Concisely, all low quality sequences, tags, non-bacterial ribosomal sequences, and chimers were removed from the database. In total, 99,325 sequences were generated in this step. Then, the second round of sequence quality control was performed using mothur software package (Schloss et al 2009). All sequences shorter than 200 bp, or having an ambiguous base, or containing a homopolymer length equal to or greater than 8 bp were removed from the dataset. The minimum, median and maximum lengths of sequences were 221, 448 and 533bp, respectively. The unique sequences (52524 sequences) were then identified and aligned against a database of high quality 16S rRNA bacterial sequences derived from Silva (version 106) (Pruesse et al 2007). Through screening, filtering, and pre-clustering processes, columns containing a gap were removed

from all sequences to reduce noise from pyrosequencing data. The furthest neighbor algorithm with a cutoff of 95% similarity was used to assign sequences to operational taxonomic units (OTU). To estimate the number of species or OTU existing in the sampling assemblage, Chao1 and ACE richness indices, were calculated using following equations:

$$S_{chao1} = S_{obs} + \frac{n_1 (n_1 - 1)}{2(n_2 + 1)}$$

(S_{chao1} = the estimated richness, S_{obs} = the observed number of species, n_1 = the number of OTUs with only one sequence, n_2 = the number of OTUs with only two sequences).

$$S_{ACE} = S_{abund} + \frac{S_{race}}{C_{ACE}} + \frac{n_1}{C_{ACE}} \hat{\gamma}^2_{ACE}, \hat{\gamma}^2_{ACE} < 0.80$$

or $$S_{ACE} = S_{abund} + \frac{S_{rare}}{C_{ACE}} + \frac{n_1}{C_{ACE}} \gamma^2_{ACE}, \gamma \geq 0.80 \quad (\quad N_{rare} = \sum_{i=1}^{abund} i n_i, \quad C_{ACE} = 1 - \frac{n_1}{N_{rare}} \quad ,$$

$$\hat{\gamma}^2_{ACE} = \max\left[\frac{S_{rare}}{C_{ACE}} \frac{\sum_{i=1}^{abund} i(i-1) n_i}{N_{rare}(N_{rare} - 1)} - 1, 0 \right],$$

$$\gamma^2_{ACE} = \max\left[\hat{\gamma}^2_{ACE} \left\{ 1 + \frac{N_{rare}(1 - C_{ACE}) \sum_{i=1}^{abund} i(i-1) n_i}{N_{rare}(N_{rare} - C_{ACE})} \right\}, 0 \right])$$

n_i = the number of OTUs with I individuals, S_{rare} = the number of OTUs with 'abund' or fewer individuals, S_{abund} = the number of OTUs with more than 'abund' individuals, abund =the threshold to be considered an'abundant' OTU).

The diversity within each individual sample, which is made up of richness and species abundance, was estimated using Simpson and non-parametric Shannon diversity indices. And following equations were used to calculate:

$H_{Shanon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} In \frac{n_i}{N}$ (S_{obs} = the number of observed OTUs, n_i = the number of individuals in OTUs, N= the total number of individuals in the community).

$D_{Simpson} = \sum_{i=1}^{S_{obs}} \frac{n_i(n_i - 1)}{N(N-1)}$ (S_{obs} = the number of observed OTUs, n_i = the number of individuals in OTUs, N= the total number of individuals in the community).

Good's non-parametric coverage estimator was used to estimate the percentage of the total species that was sequenced in each sample by using equation: $C = 1 - \frac{n_i}{N}$ (n_i = the number of OTUs that have been sampled once, N= the total number of individuals in the sample).

Rarefaction curves for treatment groups were created in Mothur, based on a re-sampling without replacement approach. Representative sequences from each OTU were taxonomically classified with a confidence level of 60% using RDP Bayesian approach (Wang et al 2007).

4.3.4 Statistical analysis

The percentage data approach was used to evaluate statistical differences among treatments at the phylum and genus taxonomical levels. Using this technique, the count data for each taxon was first transformed to the percentage of that taxon in an individual sample. The normal distribution of residuals of each phylum and genus was tested using UNIVARIATE procedure of SAS (SAS Institute Inc., 2008). For data that were not normally distributed, Poission and negative binomial distribution model in GLIMMIX procedure of SAS (ver 9.2; SAS Institute Inc., 2008) were then used to assess the effect of treatments. All taxa above 1% of the population were considered abundant and those below

1% were classified as low-abundance. The differences between treatments were considered significant at $P < 0.05$.

To identify the effects of treatments, partial least square discriminant analysis (PLS-DA; SIMCA-P+ 12.0.1, Umetrics, Umea, Sweden) was performed on genus data (when sequences were unclassified at the genus level, they have been labeled based on the upper taxonomical level). The percentage genera data were Unit Variance scaled in SIMCA-P+. Cross-validation was used to determine the number of significant PLS components and the model was validated by permutation testing. To avoid over parameterization of the model, variable influence on projection value (VIP) was estimated for each genus. If estimated VIP of genera were smaller than 0.35, this genera would be removed from the final model because it had minor contribution to the discrimination of the treatments in a first PLS-DA (VIP < 0.35) (Pérez-Enciso and Tenehaus 2003, Verhulst et al 2011).

4.4 Results

4.4.1 Bacterial α-diversity

The summary statistics of pyrosequencing 16S rRNA sequences is shown in Table 1. The WM had the highest OTU and Chao1 richness (2755, 4870, respectively; $P = 0.05$). However, other richness and diversity estimators were not significantly different under different cropping practices. Compared to CT, ZT had numerically higher OTU, richness and diversity (Table 4.1). The rarefaction curve (Figure 4.1 and 4.2) generated with mothur showed that the observed number of OTU of WM was highest among all of cropping systems. The curve at 95% distance, corresponding to genus level of diversity, showed 5000 OTUs in soil under WM whereas around 4000 OTUs were observed in soil under

other cropping systems (including WR, CR, CM, ZT and CT) for the same number of sequences reads (15000). In addition, all rarefaction curves were not saturated.

4.4.2 Bacterial community composition

The 91,202 sequences were affiliated with14 phyla, of which seven were abundant (> 1% of population) including Proteobacteria, Actinobacteria, Acidobacteria, Gemmatinomadetes, Bacteroidetes, Chloroflexi, and Planctomycetes. Within Proteobacteria, four classes of Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Deltaproteobacteria were abundant. The percentage of phyla Firmicutes, Fibrobacteres, Nitrospirae, Verrucomicrobia, P10, TM7 and WS3 were lower than 1% of population (Table 4.2). The CR contained a higher percentage of Proteobacteria (49.2%) and lower Actinobacteria (32.1%) compared to CM, WM and WR, although the difference was not statistically significant. The percentage of Alphaproteobacteria under CR was 25.8%, which was significantly higher than that in WM (18.0%), CM (15.4%) and WR (15.1%). In contrast, WR and WM supported a higher percentage of Deltaproteobacteria (2.8% and 2.5%, respectively) than CM and CR (1.2% and 1.0%, respectively). The percentage of phylum Gemmatinomadetes was highest under WR (5.3%), followed by WM (4.4%), CM (4.1%), and CR (1.5%). Wheat monoculture and rotation also supported a higher percentage of the phylum Chloroflexi, WS3, and Nitrospirae than canola monoculture and rotation. The percentage of TM7 was lower under wheat monoculture (0.03%) than other cropping practices. Other phyla did not show significant shift under monoculture and rotation practices. The phylum composition was similar between ZT and CT with the exception of Firmicutes, which was higher in ZT soil (5.56%).

The relative abundance of different genera belonging to the phyla Proteobacteria, Actinobacteria and Acidobacteria varied under different monoculture and rotation cropping practices (Table 4.3, 4.4 and 4.5). We found that crop (canola *vs.* wheat) rather than treatment (monoculture *vs.* rotation) was the main factor, which influenced the relative abundance of genera. Wheat was associated with a higher percentage of *Blastococcus*, *Arthrobacter*, *Pseudonocardia*, and *Solirubrobacter*, while canola supported relatively higher populations of *Brevundimonas, Pesudolabrys, Variovarax* and *Koribacter*. Different tillage practices did not significantly influence the relative abundance of different genera.

The PLS-DA analysis showed that there is a significant difference in bacterial communities between canola and wheat. Furthermore, bacterial communities were similar under WM and WR, while significant differences were observed between CM and CR (three latent variables, R^2X_{cum} = 0.496, R^2Y_{cum}= 0.780, Q^2_{cum}= 0.11) (Figure 4.3). Phylum, Family and Genera that were the most characteristic of each management system were identified using a loading-plot (Figure 4.5). Among the bacterial taxa that were included in the model, *Blascoccus, Solirubrobacter, Pseudonocardia,* Gemmatimonadaceae (F), Acidobacteriaceae (F), Micromonosporaceae (F), and Planctomycetes (P) were highly correlated with wheat cropping systems. In contrast, *Pseudolabrys*, *Brevundimonas* and *Koribacter* were correlated with canola monoculture, while *Variovorax* was significantly correlated with canola rotation (Figure 4.5 and Figure 4.7). Figure 4.4 showed that there was a significant difference in bacterial communities between ZT and CT (three latent variables: R^2X_{cum} =0.834, R^2Y_{cum}=0.991, Q^2_{cum}=0.874). By using PLS-DA loading plot and regression coefficients, the genera that was most characteristic for either treatment was also identified (Figure 4.6 and Figure 4.8). *Skemanella* significantly correlated with ZT, whereas, Chloroflexi significantly correlated with CT (Figure 4.6 and Figure 4.8).

Table 4.1 Summary statistics of pyrosequencing 16S rRNA sequences of samples

Crop	Treatment	Mean (SEM) results for indicated variable						
		Number of trimmed sequences	OTU[1] (95% distance)	Coverage (%)	Richness[2]		Diversity[3]	
					Chao1	ACE	Shannon	Simpson
Canola	Monoculture	15,116	1581.7[b]	83.2	2928.8[b]	3842.5	6.78	0.003
Canola	Rotation	14,604	1540.7[b]	82.4	3047.4[b]	4197.4	6.75	0.004
Wheat	Monoculture	13,254	2755.0 [a]	81.1	4870.8[a]	5808.4	7.78	-0.002
Wheat	Rotation	16,025	1702.0[b]	82.7	3081.9[b]	4051.8	6.82	0.004
SEM			249.97	0.84	379.36	521.36	0.28	0.0018
Crop (P- value)			0.05	0.63	0.05	0.11	0.12	0.15
Management (P- value)			0.09	0.82	0.09	0.15	0.14	0.14
Crop x Management			0.11	0.17	0.07	0.09	0.17	0.21
Conventional tillage		15,116	1581.7	83.2	2928.8	3842.5	6.78	0.003
Zero tillage		23,208	2141.7	85.1	3947.2	5359.9	6.99	0.004
SEM			271.77	0.01	469.13	592.17	0.34	0.005
P- Value			0.22	0.33	0.19	0.18	0.81	0.92

[a,b,c] Means with different letters are significantly different for management at P< 0.05.

[1] OTU= operational taxonomic units

[2] Based on Chao1 and abundance based coverage estimation (ACE) richness indices

[3] Based on Shannon and Simpson diversity estimators

Table 4.2 Phylogenetic composition of bacterial phyla from pyrosequenced 16S rRNA sequences

Phylum	Canola		Wheat		SEM	*P*-Value			ZT	CT	SEM	*P*-Value
	Monoculture	Rotation	Monoculture	Rotation		Crop	Treat	Crop x Treat				
	……Abundant phylum……..											
Proteobacteria	30.9	49.2	31.2	32.8	4.3	0.11	0.06	0.10	34.3	30.9	2.87	0.34
Alphaproteobacteri	15.4[b]	25.8[a]	18.0[a,b]	15.1[b]	2.71	0.13	0.16	0.03	15.6	15.4	4.63	0.47
Betaproteobacteria	9.5	11.9	6.5	7.5	1.69	0.07	0.34	0.70	15.6	9.5	2.72	0.23
Gammaproteobacte	4.7	10.4	4.2	7.3	2.27	0.46	0.10	0.58	4.8	4.7	0.74	0.50
Deltaproteobacteria	1.2[b]	1.0[b]	2.5[a]	2.8[a]	0.44	0.01	0.96	0.56	0.5	1.2	0.22	0.54
Actinobacteria	44.8	32.1	42.9	35.4	6.31	0.90	0.11	0.64	42.8	44.8	3.28	0.45
Acidobacteria	7.0	4.9	8.7	11.8	2.33	0.11	0.83	0.31	7.3	6.97	3.19	0.91
Gemmatinomadetes	4.1[a]	1.5[b]	4.4[a]	5.3[a]	0.94	0.04	0.18	0.04	2.2	4.08	1.05	0.31
Bacteroidetes	3.8	7.4	1.8	3.2	1.55	0.09	0.16	0.52	4.4	3.84	2.24	0.62
Chloroflexi	3.2[a,b]	1.6[b]	5.6[a]	5.2[a]	0.90	0.02	0.30	0.56	3.7	3.20	1.34	0.77
Planctomycetes	1.3	0.8	1.5	2.1	0.39	0.11	0.97	0.26	1.3	1.29	0.49	0.80
	……Low-abundant phylum……											
Firmicutes	1.3	0.9	0.9	1.0	0.59	0.25	0.35	0.24	5.6	1.30	1.09	0.10
Fibrobacteres	0.3	0.03	0.3	0.2	0.22	0.41	0.22	0.26	0.1	0.29	0.11	0.44
Nitrospirae	0.3[a,b]	0.2[b]	0.4[a]	0.4[a]	0.07	0.02	0.61	0.52	0.3	0.23	0.12	0.29
Verrucomicrobia	0.3	0.3	0.5	0.8	0.38	0.06	0.52	0.35	0.4	0.32	0.11	0.58
P10	0.6	0.4	0.3	0.5	0.13	0.55	0.86	0.15	0.3	0.55	0.17	0.13
TM7	0.1[a]	0.2[a]	0.03[b]	0.1[a]	0.04	0.02	0.50	0.96	0.1	0.12	0.03	0.30
WS3	0.03[b]	0.01[b]	0.1[a]	0.1[a]	0.14	0.05	0.74	0.81	0.03	0.03	0.02	0.92
	……Unclassified……											
Unclassified	1.6	0.9	1.8	1.7	0.24	0.13	0.21	0.29	1.2	1.59	0.33	0.16

[a] [a,b,c] Means within the same row different is significant (P<0.05)

Core Phyla is percentage of sequences is larger than 1, Non-core Phyla is percentage of sequences is smaller than 1.

* Percentage is <0.1

Table 4.3 Phylogenetic composition of bacterial genera in *Actinobacteria* generated using pyrosequenced 16S rRNA sequences

Family; Genus	Canola		Wheat			*P*-Value			ZT	CT	SEM	*P*-Value
	Monoculture	Rotation	Monoculture	Rotation	SEM	Crop	Treat	Crop x Treat				
Nocardiaceae; Rhodococcus	1.7	1.3	0.1	0.1	0.81	0.11	0.83	0.83	2.4	1.67	1.43	0.71
Geodermatophilaceae; Blastococcus	0.9[b]	0.4[b]	2.0[a]	1.7[a]	0.25	0.002	0.15	0.68	0.6	0.70	0.22	0.96
Microbacteriaceae; Frigoribacterium	2.8	1.0	0.3	0.5	0.93	0.34	0.58	0.51	3.6	2.83	2.18	0.79
Micrococcaceae; Arthrobacter	1.9[b]	1.3[b]	3.4[a]	3.2[a]	0.55	0.01	0.58	0.83	2.3	1.94	0.62	0.66
Micromonosporaceae; Actinoplanes	0.3	1.1	1.0	0.8	0.34	0.53	0.31	0.16	0.2	0.28	0.08	0.98
*Micromonosporaceae*_unclassified	0.7[b]	0.9[b]	1.8[a]	1.5[a,b]	0.21	0.01	0.44	0.41	0.5	0.69	0.14	0.64
Nocardioidaceae; Marmoricola	1.6	1.2	1.6	0.5	0.41	0.49	0.13	0.46	1.4	1.12	0.55	0.69
Nocardioidaceae; Nocardioides	1.4	2.5	2.1	1.1	0.77	0.67	0.93	0.23	1.4	1.11	0.67	0.75
Propionibacteriaceae; Microlunatus	1.2	0.9	1.8	1.6	0.32	0.10	0.41	0.91	1.4	0.93	0.22	0.15
Pseudonocardiaceae; Pseudonocardia	1.0[b]	0.7[b]	1.4[a]	1.6[a]	0.15	0.01	0.88	0.19	0.7	0.65	0.09	0.46
Streptomycetaceae; Streptomyces	0.8	0.3	0.6	0.7	0.17	0.71	0.20	0.12	0.7	0.66	0.1	0.82
Solirubrobacteriaceae; Solirubrobacter	0.7[b]	0.5[b]	1.9[a]	1.3[a,b]	0.24	0.02	0.07	0.88	0.9	0.69	0.14	0.33
Intrasporangiaceae; unclassified	0.9	0.4	0.4	0.4	0.35	0.45	0.48	0.53	0.7	0.91	0.48	0.78

[a,b,c] Means within the same row difference is significant (P<0.05)

Table 4.4 Phylogenetic composition of bacterial genera in *Proteobacteria* generated using pyrosequenced 16S rRNA

Class; Family; Genus	Canola		Wheat		SEM	*P*-Value			ZT	CT	SEM	*P*-Value
	Monoculture	Rotation	Monoculture	Rotation		Crop	Treat	Crop X Treat				
Alphaproteobacteria	15.4[b]	25.8[a]	18.0[a,b]	15.1[b]	2.71	0.13	0.16	0.03	15.6	15.4	4.63	0.47
Caulobacteraceae; Brevundimonas	1.8[a]	0.4[b]	0.3[b]	0.2[b]	0.35	0.02	0.06	0.04	1.1	0.4	0.9	0.18
Bradyrhizobiaceae; Bradyrhizobium	0.4	0.8	1.2	0.7	0.22	0.17	0.58	0.09	0.3	0.3	0.07	0.58
Hyphomicrobiaceae; Devosia	0.4	2.3	1.0	0.4	0.46	0.13	0.13	0.02	1.2	0.4	0.73	0.84
Rhizobiaceae; Rhizobium	0.3[b]	3.5[a]	0.3[b]	0.3[b]	1.01	0.18	0.15	0.16	0.7	0.3	0.27	0.36
Xanthobacteraceae; Pseudolabrys	1.9[a]	0.8[b]	0.6[b]	0.7[b]	0.37	0.05	0.19	0.09	1.0	1.9	0.51	0.37
*Xanthobacteraceae*_unclassfied	0.7	0.6	1.1	0.9	0.21	0.26	0.22	0.77	0.5	0.7	0.23	0.73
*Acetobacteraceae*_unclassified	0.8	1.1	1.0	0.9	0.36	0.60	0.85	0.81	1.0	0.8	0.22	0.64
Betaproteobacteria	9.5	11.9	6.5	7.5	1.69	0.07	0.34	0.70	15.6	9.5	2.72	0.23
Alcaligenaceae; Achromobacter	0.1	2.2	0.01	0.1	0.90	0.26	0.26	0.28	0.2	0.1	1.84	0.62
Comamonadaceae; Methylibium	0.8	1.4	1.0	1.7	0.45	0.86	0.29	0.62	1.1	0.8	0.15	0.18
Comamonadaceae; Variovorax	0.4[b]	1.5[a]	0.3[b]	0.5[b]	0.18	0.02	0.01	0.04	0.8	0.4	0.27	0.41
Oxalobacteraceae; Massilia	1.9	2.4	0.2	0.3	0.96	0.22	0.85	0.87	5.0	1.9	2.29	0.38
*Oxalobacteraceae*_unclassified	0.9	0.4	0.7	0.5	0.45	1.00	0.47	0.79	3.5	0.9	0.66	0.23
*Nitrosomonadaceae*_unclassified	0.7	0.5	0.4	0.7	0.23	0.45	0.98	0.16	0.4	0.7	0.31	0.55
Gammaproteobacteria	4.7	10.4	4.2	7.3	2.27	0.46	0.10	0.58	4.8	4.7	0.74	0.50
Pseudomonadaceae; Pseudomonas	0.5	1.5	0.4	2.4	0.75	0.73	0.22	0.69	1.2	0.5	0.68	0.41
*Sinobacteraceae*_unclassified	0.3	0.6	1.3	0.9	0.49	0.12	0.70	0.37	0.2	0.3	0.04	0.57
Xanthomonadaceae; Rhodanobacter	1.6	0.8	0.3	0.6	0.46	0.06	0.54	0.12	1.2	1.6	0.53	0.88
Xanthomonadaceae; Stenotrophomonas	0.2	4.5	0.2	0.1	0.48	0.10	0.11	0.10	0.3	0.2	1.19	0.85
Deltaproteobacteria	1.2[b]	1.0[b]	2.5[a]	2.8[a]	0.44	0.01	0.96	0.56	0.5	1.2	0.22	0.54

[a,b,c] Means within the same row difference is significant (P<0.05)

Table 4.5 Phylogenetic composition of bacterial genera in *Acidobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, Planctomycetes* generated using pyrosequenced 16S rRNA sequences

Family; Genus	Canola		Wheat			*P*-Value						
	Monoculture	Rotation	Monoculture	Rotation	SEM	Crop	Treat	Crop x Treat	ZT	CT	SEM	*P*-Value
……..Phylum ***Acidobacteria***……												
Acidobacteriaceae; *Koribacter*	1.6^{a}	1.1^{a}	0.2^{b}	0.2^{b}	0.43	0.04	0.60	0.54	0.4	1.6	0.72	0.39
*Acidobacteriaceae*_unclassified	1.5^{b}	2.4^{b}	7.6^{a}	8.9^{a}	1.60	0.04	0.81	0.74	1.5	1.5	0.57	0.98
……..Phylum ***Bacteroidetes***……												
Flavobacteriaceae; *Flavobacterium*	0.8	2.5	0.7	0.8	0.67	0.16	0.17	0.21	1.1	0.8	0.57	0.75
*Chitinophagaceae*_unclassified	0.6	0.7	0.3	1.0	0.22	0.92	0.06	0.10	0.5	0.6	0.15	0.87
Sphingobacteriaceae; *Pedobacter*	1.7	2.6	0.1	0.3	0.97	0.06	0.50	0.71	6.3	1.7	2.51	0.27
……..Phylum ***Chloroflexi***……												
*Chloroflexi*_unclassified	0.7	0.5	1.0	0.7	0.16	0.21	0.18	0.71	0.4	0.7	0.09	0.09
……Phylum ***Firmicutes***……												
Bacillaceae; *Bacillus*	0.9	0.5	0.5	0.6	0.21	0.43	0.45	0.23	0.9	0.9	0.46	0.97
……Phylum ***Gemmatimonadetes***……												
Gemmatimonadaceae;	1.6	0.7	1.7	1.9	0.60	0.84	0.24	0.14	1.5	1.6	0.47	0.90
*Gemmatimonadaceae*_unclassified	0.9^{b}	0.5^{b}	2.4^{a,b}	3.0^{a}	0.51	0.01	0.58	0.10	0.5	0.9	0.34	0.48
……..Phylum ***Planctomycetes***……												
*Planctomycetes*_unclassified	0.6^{B}	0.4^{B}	0.7^{A,B}	1.3^{A}	0.18	0.07	0.60	0.02		0.6		

^{a,b,c} Means within the same row difference is significant (P<0.05)

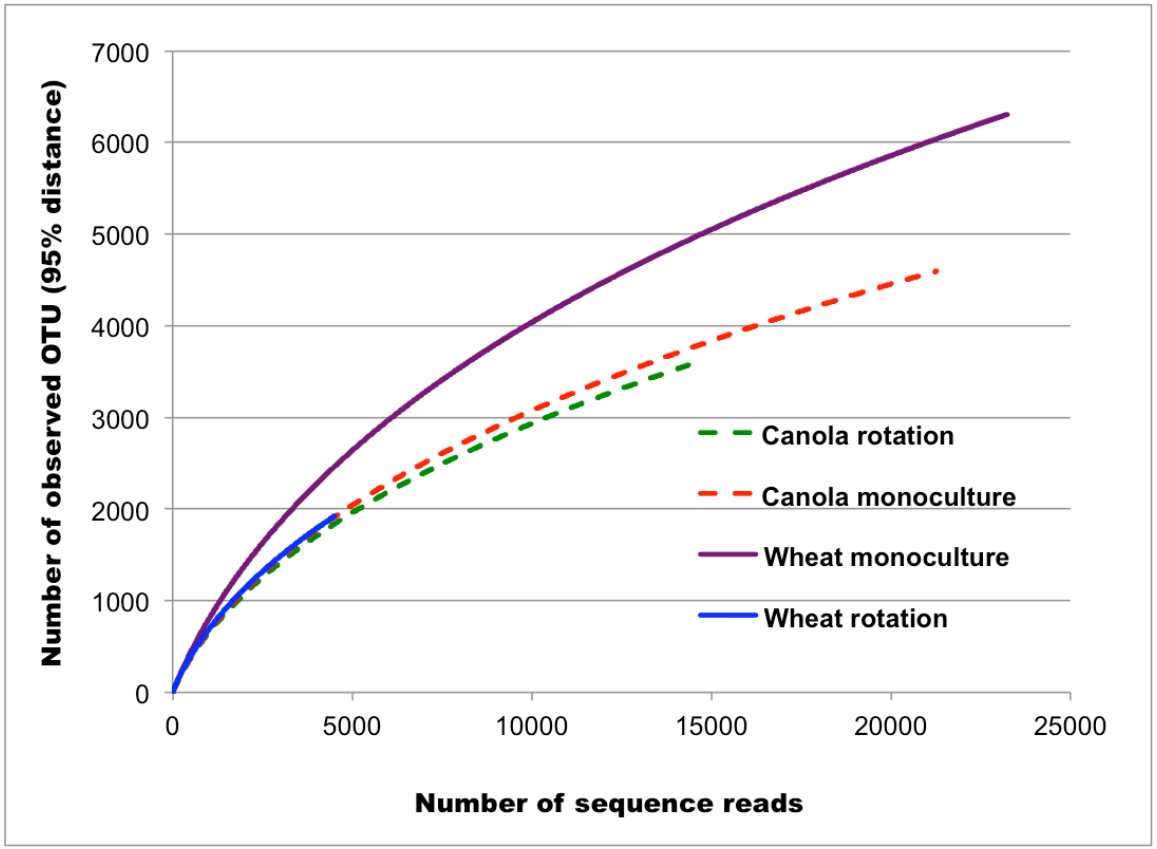


Figure 4.1 Rarefaction curves of monoculture vs. rotation treatment samples at OTU cutoff of 0.05 distance.

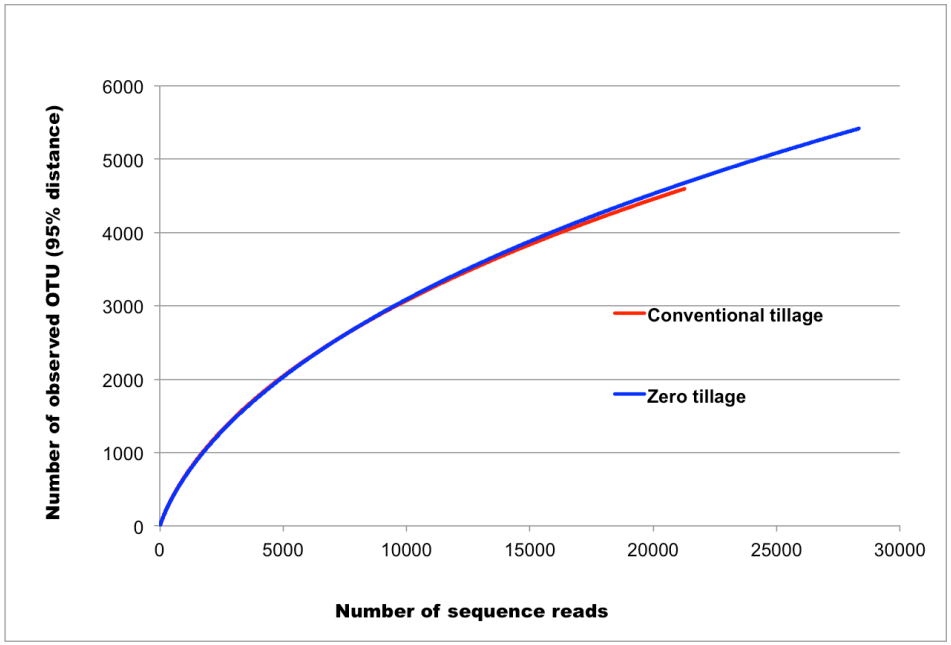


Figure 4.2 Rarefaction curves of Zero tillage vs. Conventional tillage treatment samples at OTU cutoff of 0.05 distance.

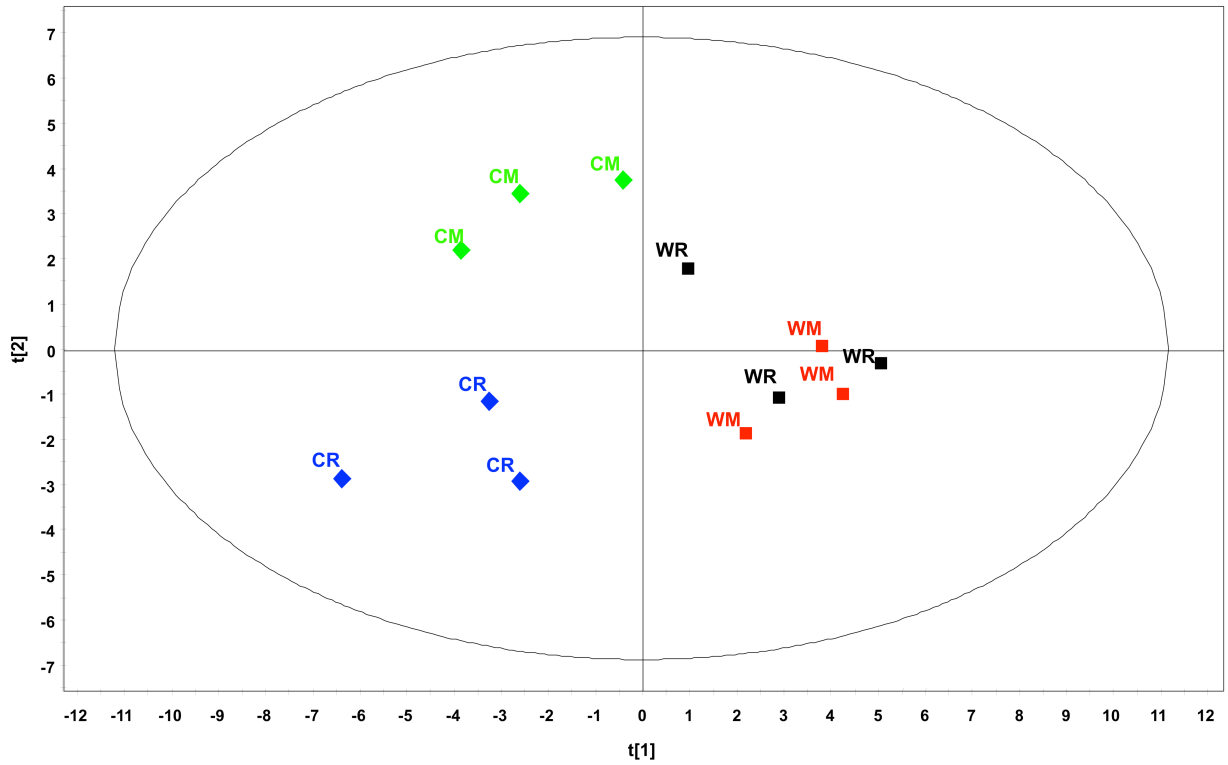


Figure 4.3 Partial least square discriminant score plot of soil bacteria under rotation and monoculture treatments. CM: Canola monoculture; CR: Canola rotation; WM: Wheat monoculture; WR: Wheat rotation.

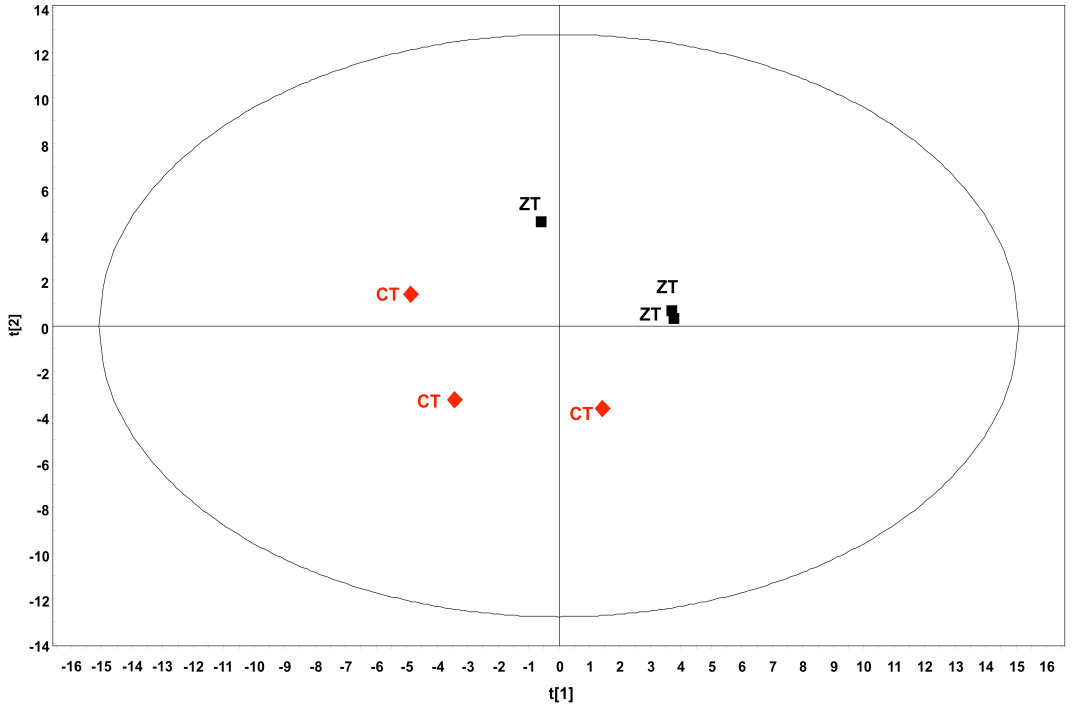


Figure 4.4 Partial least square discriminant score plot of soil bacteria under zero tillage and conventional tillage. ZT: zero tillage, CT: conventional tillage

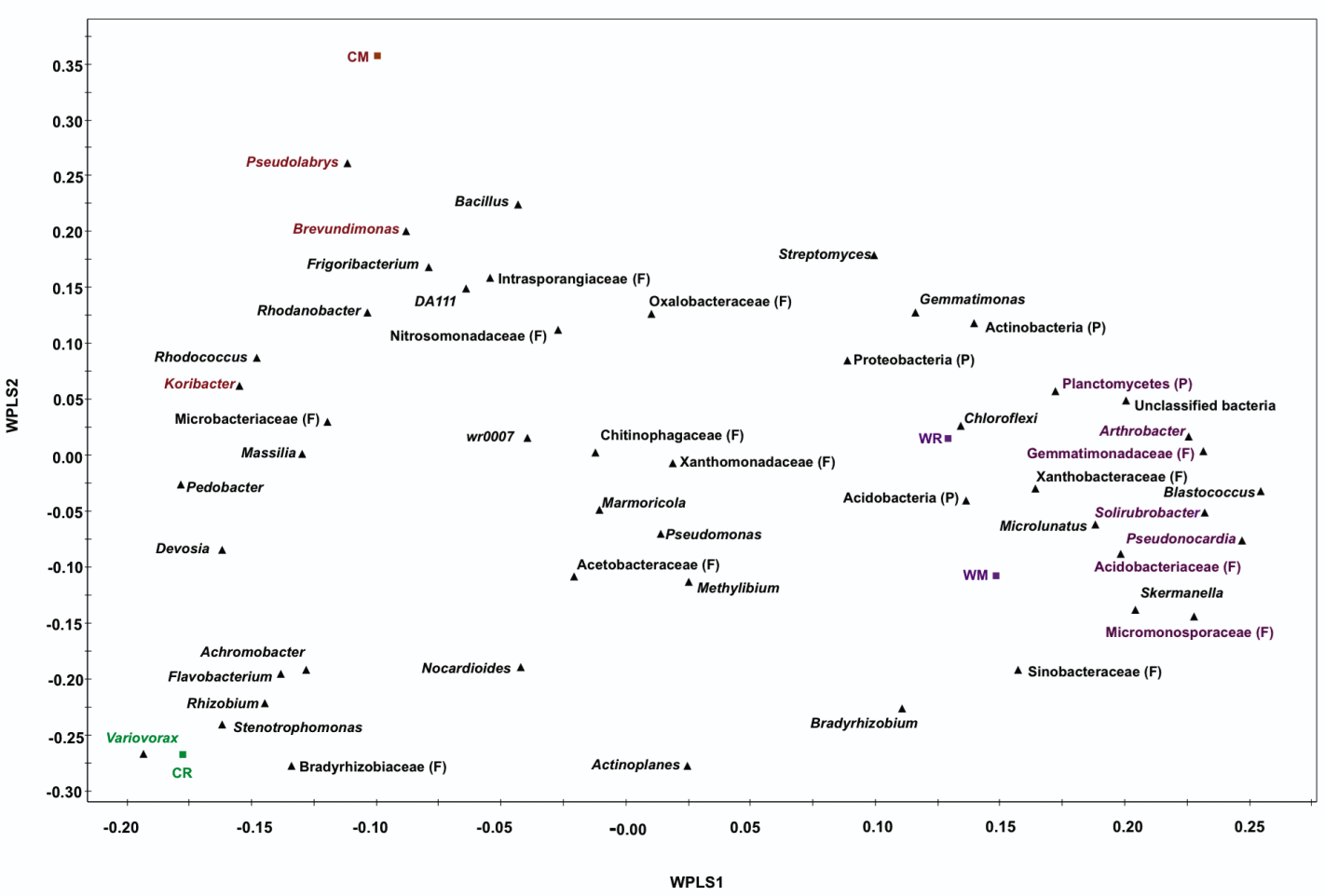


Figure 4.5 Partial least square discriminant analysis (PLS-DA) of the bacterial genera associated with rotation and monoculture treatments**.** PLS-DA loading plot based on the relative abundance of bacterial genera in the microbial community profiles of different cropping treatments.

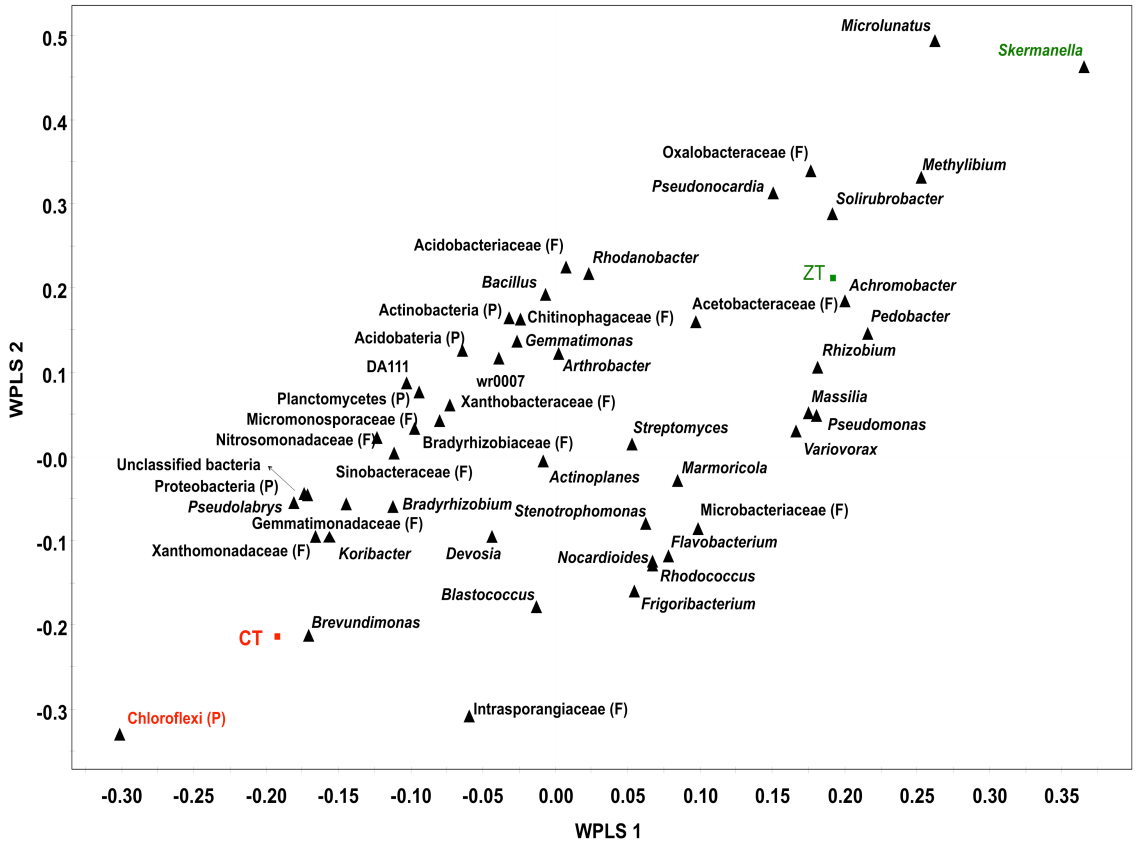


Figure 4.6 Partial least square discriminant analysis (PLS-DA) of the bacterial genera associated with zero tillage and conventional tillage treatments.

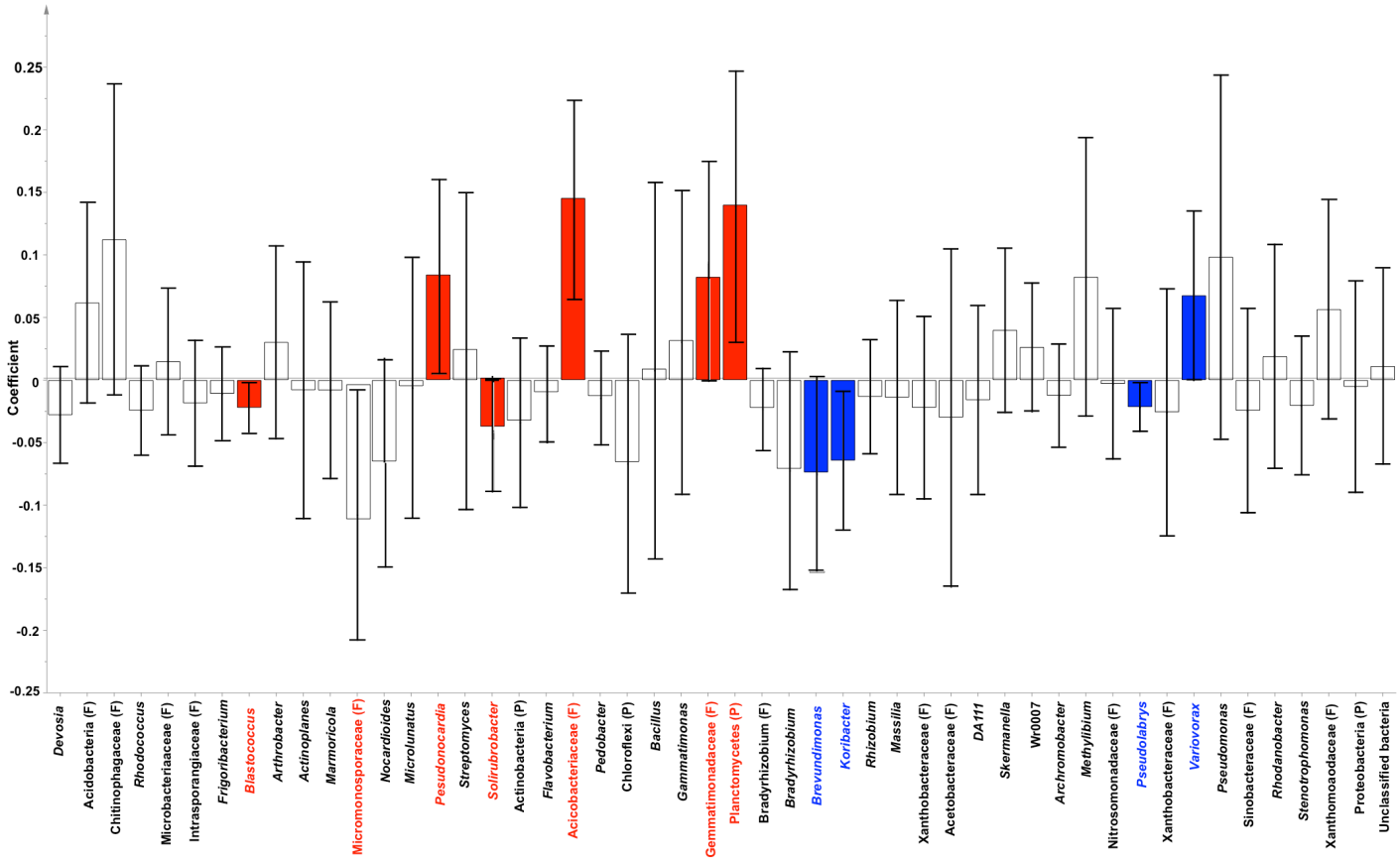


Figure 4.7 Coefficient plot of the bacterial profiles of rotation and monoculture treatments. Partial least squares discriminant analysis (PLS-DA) coefficient plot based on the relative abundance of the bacterial genera in the microbiome profile of rotation and monoculture treatments. Genera with significantly positive (>0) or negative (<0) PLS regression coefficients (i.e. no overlap between the 95% confidence interval indicated and the horizontal axis) contribute significantly to the prediction of the wheat (red bar) or canola (blue bar) samples.

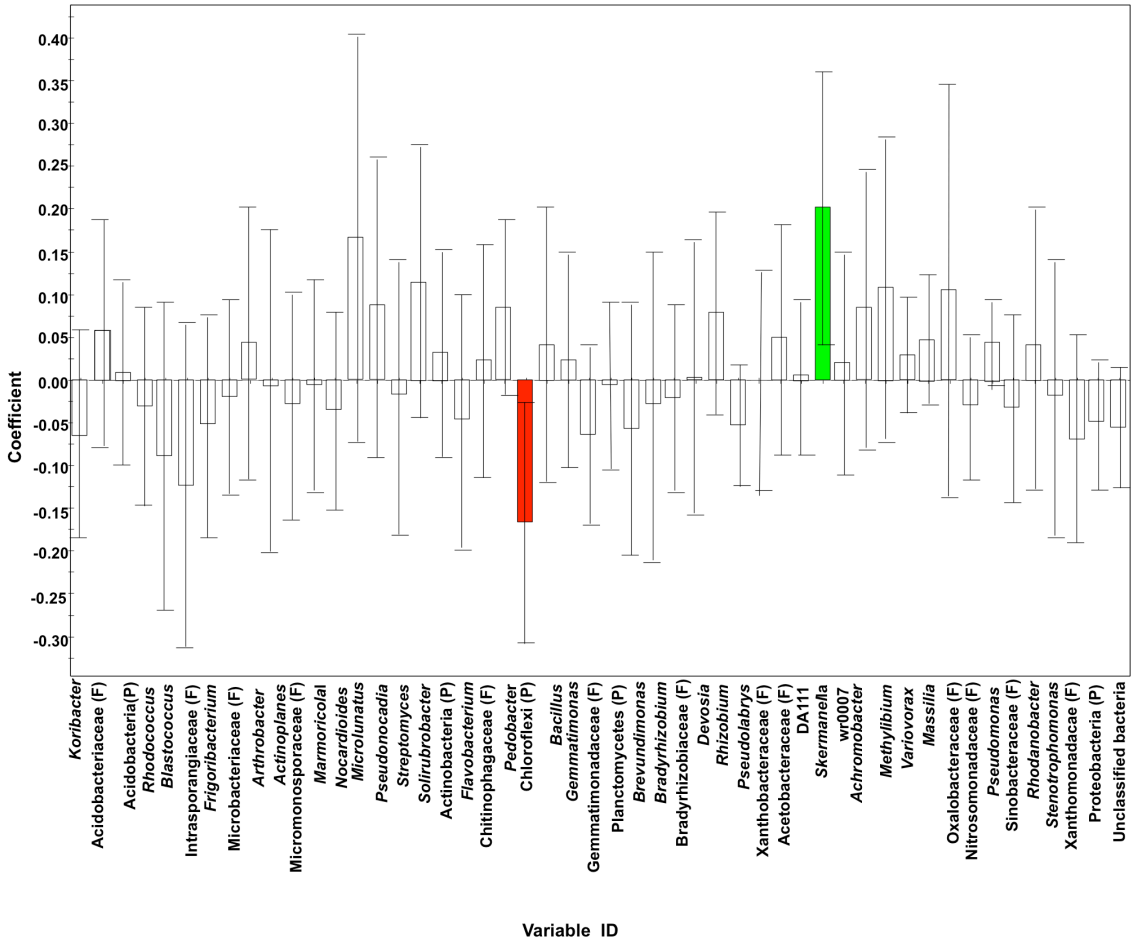


Figure 4.8 Coefficient plot of the bacterial profiles of tillage treatments. Partial least squares discriminant analysis (PLS-DA) coefficient plot based on the relative abundance of the bacterial genera in the microbiome profile of tillage treatments. Genera with significantly positive (>0) or negative (<0) PLS regression coefficients (i.e. no overlap between the 95% confidence interval indicated and the horizontal axis) contribute significantly to the prediction of the conventional tillage (red bar) or zero tillage (green bar) samples.

4.5 Discussion

In this study we found that bacterial community structure in the soil was primarily affected by plant species and to varying degrees by farming practices, such as rotation, monoculture or tillage. Our results showed that wheat monoculture had the highest numbers of OTU, as well as richness and diversity, compared to wheat rotation, canola monoculture and canola rotation (Table 4.1). Yin et al (2010) have reported similar results indicating richness was higher in soil continuously growing wheat, while diversity was not influenced by wheat monoculture or wheat-soybean rotation treatment. The importance of plant species for the bacterial community structure was evident in the experiment with canola and wheat (Figure 4.3 and Figure 4.5). Canola harbored a relatively lower percentage of Deltaproteobacteria, Chloroflexi, Nitrospirae, Planctomycetes and WS3 phyla, and a lower percentage of *Blastococcus*, *Arthrobacter, Pseudonocardia*, *Solirubrobater* genera compared to wheat (Table 4.2 and 4.3). The difference of crop root exudation could result in the apparent difference in soil bacterial communities (Marschner et al 2004). Canola root could release S-containing compounds, glucosinolates and isothiocyanates, which have the ability to inhibit the growth of some microorganisms, including soil-borne pathogens (Brown and Morra, 1997, Kirkegaard et al 2001, Rumberger and Marschner, 2002; Pascault 2010). On the other hand, our study shows that canola supports a relatively higher percentage of specific genera including *Brevundimonas*, *Pseudolabrys, Rhodanobater,* and *Koribacter*. The genus *Rhodanobacter,* which belongs to Acidobacteria, has denitrification capability (Kostka et al 2012). As such, growing canola in large scale could influence the nitrogen cycle in the environment. Moreover, crop could be modified with respect to the treatments (rotation *vs.* monoculture) (Figure 4.3, 4.5 and Figure 4.7). Canola rotation was

associated with a higher percentage of *Stenotrophomonas*, *Rhizobium,* and *Pseudomonas* genera (Table 4.4). Many species in *Pseudomonas* and *Stenotrophomonas* play important roles as biological control agents against soil-borne plant pathogens (Garbeva et al 2004, Ryan et al 2009). For example, some *Stenotrophomonas* spp. (*S. maltophilia*) can protect plants through producing antimicrobial compounds as well as promote plant growth (Messiha 2007). Also, many *Stenotrophomonas* are capable of degrading a wide range of compounds, including pollutants (Liu et al 2007; Chen et al 2008). Thus, they could potentially be used as plant-growth promoting rhizobacteria (PGPR), or in bioremediation and phytoremediation. Certain members of *Pseudomonas* genus (such as *P. fluorescence* strains CHA0 or Pf-5, *P. chlororaphis* strain PA23) have shown the ability to control crop pathogens (Haas and Défago 2005; Nakkeeran et al 2006). Previous studies have shown there is a difference in microbial communities harbored by wheat and canola by using fatty acid methyl ester (FAME) analysis (Germida et al 1998); however, the resolution of this method is low. To the best of our knowledge, this is first comprehensive study showing soil bacterial community differences in canola- and wheat- associated soil. Therefore, we think that integrating canola into a crop system could increase beneficial bacterial communities.

We only assessed the bacterial communities and did not evaluate other microbial communities, such as fungi and archaea. Some studies showed the *Arbuscular mycorrhizal* fungi (AMF), which colonize plant root systems, could get engaged in shaping the soil bacterial population too (Toljander et al 2007). Canola is a non-AMF host crop. As such, canola monoculture would result in the low abundance of AMF. In contrast, canola rotation with wheat, pea and oat, which are crops associated with AMF, are expected to change the abundance of AMF in the soil. Therefore, the bacterial communities under the canola monoculture and canola rotation showed significant differences between them (Figure 4.3

and Figure 4.5). The bacterial communities were more similar under wheat monoculture and wheat rotation, compared to those in canola monoculture and canola rotation. This could be due to consistent presence of AMF communities dwelling in the soil of wheat cropping systems; however this has yet to be established.

Our experimental trials could allow us to test the effect of a single factors namely tillage, on the bacterial communities under canola monoculture. Based on the SAS analysis, the OTU number in rarefaction curve (Figure 4.2), richness index of Chao1 and ACE, and diversity index of Simpson and Shannon of bacterial communities were found to be slightly higher under ZT treatment (Table 4.1). The higher number of trimmed sequences of ZT could result in this. Furthermore, we investigated the individual bacterial percentage under different tillage systems (Table 4.2, 4.3, 4.4 and 4.5), and found that the percentage of bacteria was similar, except of Firmicutes and Chloroflexi, which showed a different trend at P=0.1. These results were consistent with other studies. By using phospholipid fatty acid analysis (PLFA) and denaturing gradient gel electrophoresis (DGGE), Helgason et al. (2010) investigated long-term no-till management effect on microbial communities in Canadian prairie agro ecosystems. They found that differences in bacterial communities were related to depth in the soil profile rather than tillage management. Wortmann et al (2008) also found that bacterial communities in the 0- to 30- cm depth, on a mass equivalent basis, were not significantly affected by tillage. Our PLS-DA multivariate analysis showed that bacterial communities were influenced by tillage (Figure 4.4, 4.6 and Figure 4.8), since the bacterial communities under ZT were separate from CT. Differences in the results obtained using the classical SAS analysis compared to PLS-DA are most likely due to the nature of these methodologies. Classical statistical analyses are usually conservative when there are only few observations. In addition, they compare only one

variable under different treatments. In contrast, PLS-DA is designed specifically for the analysis of "omics" data where there are usually a smaller number of replicates, and the number of variables is more than the number of observations. In contrast to classical statistical analysis, PLS-DA looks at all of the variables together, avoids the loss of information and attempts to find underlying relationships between whole bacterial communities and tillage treatment through the projection process. Therefore, the PLS-DA method is more suitable for analysis of the 'omics' data collected in the current study.

Our results showed canola monoculture shifts the bacterial communities significantly, compared to canola rotation. Meanwhile, integrating canola into wheat cropping systems may increase some beneficial bacterial populations, such as *Pseudomonas, Stenotrophomonas*, and *Rhizobium.* These bacterial populations could help protect crops against pathogens dwelling in the soil. Our research also demonstrated that bacterial community structure did not shift significantly by different tillage under canola monoculture. However, further investigations are need to be conducted to examine the shift of fungal, especially AMF communities and pathogen populations under these treatments, and to get comprehensive insight on the structure and function of microbial communities. Ultimately, more productive, sustainable canola and wheat farming systems will be developed based on a better understanding of how cropping systems affect soil beneficial bacterial communities.

5.0 PYROSEQUENCING REVEALS THE INFLUENCE OF ORGANIC AND CONVENTIONAL FARMING SYSTEMS ON BACTERIAL COMMUNITIES[1]

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5.1 Abstract

It has been debated how different farming systems influence the composition of soil bacterial communities, which are crucial for maintaining soil health. In this research, we applied high-throughput pyrosequencing of V1 to V3 regions of bacterial 16S rRNA genes to gain further insight into how organic and conventional farming systems and crop rotation influence bulk soil bacterial communities. A 2 × 2 factorial experiment consisting of two agriculture management systems (organic versus conventional) and two crop rotations (flax-oat-faba bean-wheat versus flax-alfalfa-alfalfa-wheat) was conducted at the Glenlea Long-Term Crop Rotation and Management Station, which is Canada's oldest organic-conventional management study field. Results revealed that there is a significant difference in the composition of bacterial genera between organic and conventional management systems but crop rotation was not a discriminator factor. Organic farming was associated with higher relative abundance of *Proteobacteria*, while *Actinobacteria* and *Chloroflexi* were more abundant in conventional farming. The dominant genera including *Blastococcus, Microlunatus, Pseudonocardia, Solirubrobacter, Brevundimonas, Pseudomonas*, and *Stenotrophomonas* exhibited significant variation between the organic and conventional farming systems. The relative abundance of bacterial communities at the phylum and class level was correlated to soil pH rather than other edaphic properties. In addition, it was found that *Proteobacteria* and *Actinobacteria* were more sensitive to pH variation.

5.2 Introduction

It has long been recognized that maintaining biodiversity of soil microbes is crucial to soil health, which has been defined as soil with the capacity of resilience to stress, sustaining high biological diversity, productivity and high level of internal nutrient cycling, maintaining environmental quality and promoting plant health (Doran and Parkin 1994; van Bruggen and Semenov 2000). Bacterial communities are responsible for multifaceted biological functions in soils (Dorn et al 1974; Sprent 1979; Torstensson 1980), and exert an important role in maintaining plant health (Zehnder et al 2001; Weller et al 2002; Ongena et al 2004, Suman et al 2005; Basak and Biswas 2010). In turn, soil has a direct impact on the structure and function of soil bacterial communities through perturbations caused by natural or human activities (Smalla et al 2001; Gattinger et al 2002; Upchurch et al 2008). It was reported that agricultural soil, perturbed by human activities, has different bacterial diversity, compared to non-disturbed forest and grassland soil (Roesch et al 2007; Acostamartinez et al 2008). However, there is still a lack of the detailed information about the bacterial diversity affected by agriculture perturbation.

Over the past decades, conventional agricultural management practices have involved the use of artificial chemical fertilizers and pesticides to increase crop yields. This has led to severe environmental problems such as soil degradation, emission and leaching of fertilizer and pesticide, and the emergence of pesticide resistant species (Shafiani and Malik 2003; Wasi et al 2008), resulting in an unsustainable practice (Azadi et al 2011). The aim in sustainable management systems is to maintain the biological function of the soil and to promote plant health. Organic farming contributes to these factors using techniques such as crop rotation, green manure, and biological pest control instead of chemical fertilizers and pesticides (Zhengfei 2005). Consequently, organic farming systems may

have a strong potential for restoring soil health and increase agro-ecosystem resilience to stress (Azadi et al 2011).

Few studies have evaluated the impact of fertilizer, crop rotation, and crop varieties on microbial community structure when conventional and organic farming systems are compared (Gunapala and Scow 1998; Mader et al 2002; Hartmann et al; 2006, Esperschutz et al 2007; Sugiyama et al 2010). These studies have found that fertilizers, and crop varieties and rotation could shape the size and structure of soil microbial communities. However, these studies were based on field experiments where the above-mentioned factors varied at the same time between conventional and organic soil management. Therefore, the main discriminator between conventional and organic farming could not be defined. Moreover, the results of these studies were not consistent, which could be due to different analytical methodologies that varied in resolution (Roesch et al 2007; Wu et al 2007; Joergensen et al 2010; Sugiyama et al 2010).

Previous studies in our group had investigated the effects of different agriculture management prectices (organic versus converntional) and crop rotation systems [(flax-oat-fababean-wheat (Grain-Only rotation) versus flax-alfalfa-alfalfa-wheat (Forage-Grain rotation)] on crop yield, soil edaphic traits, such as nitrate, phosphorus, pH, and organic matter. Results showed that rotation rather than farming managements affect most soil nutrient traits including nitrate-N, Oslen P, and organic matter. Whereas, farming systems affect soil pH, with lower pH in a conventional farming system than that in an organic farming system. However, the effects of farming management and rotation on bacterial community structure of the soil was not evaluated (Welsh et al 2009; Bell et al 2012).

We hypothesized that microbial composition of soil differs between organic and conventional farming sytstems or in different crop rotations. As such, some

microorganisms might be present in organic farms while absent or less frequent in conventional farms and vice versa. Similarly, there might be some bacteria that are unique to a specific crop rotation system. Using Roche 454 pyrosequencing methodology, the objective of this study was to identify bacterial populations that are associated with specific farming practice that could potentially influence soil and plant health. This manuscript provides a detailed framework of the soil bacterial composition at the genus level and its possible connection to farming practices.

5.3 Materials and Methods

5.3.1 Soil sampling, sampling site and experimental design

Soil samples were collected at Glenlea Long-term Crop Rotation and Management Station (GLCRMS) at southern Manitoba, which is Canada's longest running organic-conventional management comparison station commenced in 1992. A detailed description of the location and site management was described previously (Welsh et al 2009). In brief, the study site is located 20 km south of Winnipeg, Manitoba, Canada (N 49,39,0/ W 97,7,0). The soil type is Rego Black Chernozem and the soil texture is clay (9% sand, 26% silt, and 66% clay) with an organic matter content of 7.7%. The experiment was a randomized complete block design in a split-plot arrangement with three replicates. Two crop rotations, that is, flax-oat-fababean-wheat (Grain-Only rotation) and wheat-alfalfa-alfalfa-flax (Forage-Grain rotation) were used as main plots, and certificated organic and conventional methods served as subplots. The 2×2 combinations of treatments included: Grain-Only Organic (GO), Grain-Only Conventional (GC), Forage-Grain Organic (FO) and Forage-Grain Conventional (FC). All rotation crops appeared in the rotation each year. Subplots were 4 m by 25 m. Both organic and conventional experiments were managed

using conventional tillage and plots were tilled with a disc and a field cultivator prior to sowing. Pesticides and chemical fertilizers were applied on the conventional plots but not on the organic plots. Eighteen kg P_2O_5 was banded when wheat was seeded in conventional plots, and 65 kg Nitrogen/ha was broadcasted in conventional flax plots. One L/ha Buctril M and 0.235 L/ha Horizon were sprayed on conventional wheat plots and 0.2 L/ha Select and 1 L/ha Buctril were sprayed on conventional flax plots. These plots were managed with no external input of manure.

Bulk soil samples were randomly collected from the top level (0-15cm) throughout wheat and flax plots in June and August 2008. Part of each soil sample was kept at -20 °C prior to DNA extraction after sieving (2 mm) to remove roots and stones, while the rest was kept at 4 °C for chemical analyses. Samples were analyzed using an elemental analyzer at a commercial soil analysis laboratory (AGVISE, Northwood, ND) for total soil carbon and total soil nitrogen (Vario MAX Carbon-Nitrogen analyzer, Elemetar, Germany). Soil Carbonate carbon was analyzed with a modified pressure technique (Williams 1948). Organic matter, soil pH, and Olsen phosphorus (P_{olsen}): sodium bicarbonate-extractable phosphorus was measured as described by Welsh et al. (2007).

5.3.2 DNA extraction

To remove PCR inhibitors, such as humic acids, covalent cations and other easily dissolved organic compounds; from soil samples a pre-lysis washing procedure was introduced before DNA extraction (He et al 2005). Soil samples of 0.25g were mixed with 1.25 ml sodium phosphate (0.1 M, pH 7.5), then incubated in a shaker for 1 hr. at room temperature, followed by centrifuging for 10 min at 16000 × *g*. Supernatant was discarded. DNA was extracted from pre-washed samples using the PowerSoil DNA Isolate kit, which

included a bead-beating step, according to the manufacturer's specifications (Mobio Laboratories, Solana Beach, CA). The DNA purity and quantity were tested by using spectrophotometer (Du 800 Spectrophotometer, BECKMAN COULTER). The average ratio of 260:280 was 1.7. The average DNA yield was 10ng/μL. The variable regions of V1-V2 of the 16S rRNA genes were successfully amplified using forward primer 27F (AGAGTTTGATCMTGGCTCAG) and reverse primer 342R (CTGCTGCSYCCCGTAG), indicating that the quality of extracted DNA was sufficient for further PCR application (Khafipour et al 2009). In order to test the long-term effect of farming practices on the bacterial communities in the soil and reduce the temporal effects of different sampling times, DNA samples of the same treatment collected at different sampling times were pooled before pyrosequencing.

5.3.3 Pyrosequencing

A total of 23-pooled DNA samples were pyrosequenced using the bacterial tag-encoded GS FLX-Titanium amplicons as described by Dowd et al (2008) and Khafipour et al (2012). In brief, a mixture of Hot Start, HotStar high fidelity Taq polymerases, and Titanium reagents were used to perform a one-step PCR (35 cycles) with primers 28F (GAGTTTGATCMTGGCTCAG) and 519R (GTNTTACNGCGGCKGCTG), which covered the variable regions V1-V3 of the bacterial 16S rRNA genes (Dowd et al. 2008). The pyrosequencing procedures were carried out at the Research and Testing Laboratory (Lubbock, TX; http://www.Researchandtesting.com).

5.3.4 Bioinformatics of pyrosequencing data

5.3.4.1 Sequence editing, categorical transformation/classification

Pyrosequencing data were edited, categorically transformed and classified as described by Khafipour et al (2012). Briefly, all low quality sequences, tags, non-bacterial ribosomal sequences, and chimeras were removed from the database. In total, 123, 316 sequences were generated in this step. Then, the mothur software package (Schloss et al 2009) was utilized to perform the second round of sequence quality control and assignments of operational taxonomic units (OTU). All sequences shorter than 200bp, or sequences having one or more ambiguous base, or containing a homopolymer length equal or greater than 8bp were removed from the dataset. The minimum, median and maximum lengths of sequences were 200, 471 and 647 bp, respectively. The unique sequences were then identified and aligned against a database of high quality 16S rRNA bacterial sequences derived from Silva (version 106) (Pruesse et al 2007). Through screening, filtering, and pre-clustering processes, columns containing a gap were removed in all sequences to reduce noise from pyrosequencing data. The remaining 987 columns (with an actual sequence length varying from 203 to 342 bp) and 39,283 sequences were used to build a distance matrix with a distance threshold of 0.1. Using the furthest neighbor algorithm with a cutoff of 95% similarity, these sequences were clustered to OTU. Representative sequences from each OTU were taxonomically classified with a confidence level of 60% using RDP Bayesian approach (Wang et al 2007).

5.3.4.2 Alfa diversity analysis

An OTU-based approach was performed to calculate the richness, diversity and coverage at OTU cutoff of 0.05, which characterizes the biodiversity of the bacterial

population in the soil samples at the genus level. Richness indices, Chao1 and abundance based coverage estimation (ACE), were calculated to estimate the number of species or OTU that were present in the sampling assemblage. The diversity within each individual sample, which is made up of richness and species abundance, was estimated using Simpson and non-parametric Shannon diversity indices. Good's non-parametric coverage estimator was used to estimate the percentage of the total species that were sequenced in each sample. Rarefaction curves for treatment groups were created in mothur (Schloss et al 2009), based on a re-sampling without replacement approach.

5.3.4.3 Statistical hypothesis testing

The UNIVARIATE procedure of SAS (2008) was used to test the normality of residuals for Alfa diversity indices. Non-normally distributed data were power transformed using Box-Cox power transformation macro (http://www.datavis.ca/sasmac/boxcox.html) in SAS based on the following models: BoxCox (y) = $(y^{\lambda} - 1)/\lambda$, if $\lambda \neq 0$ OR BoxCox (y) = log (y), if $\lambda = 0$. The range of λ for each parameter was adjusted by trial and error and the best fitting value of λ was identified using maximum likelihood methods. Normalized data were then used to assess the effect of treatment using MIXED procedure of SAS (2008). The effect of replicates was treated as random in the model.

Percentage data was used to evaluate statistical differences among treatments at the phylum and genus levels. To do so, the count data for each taxon was first transformed to the percentage of that taxon in an individual sample. Then UNIVARIATE procedure of SAS was used to test the normality of residuals for percentage data at each taxonomic level. For non-normally distributed data, Poisson and negative binomial distributions were fitted in the GLIMMIX procedure of SAS (2008) to assess the effect of treatment. A log link

function was specified for Poisson and negative binomial distributions. The goodness of fit for different distributions was compared using Pearson chi-square / DF (closer to 1 is better). Taxa were categorized as abundant and low abundant in order to characterize them within each treatment. All taxa above 1% of the population were considered abundant and those below 1% were classified as less abundant (Khafipour et al 2012). The differences between treatments were considered significant at $P < 0.05$ while trends were observed at $P < 0.1$.

5.3.4.4 Partial least square discriminant analysis and redundancy analysis

Partial least square discriminant analysis (PLS-DA; SIMCA-P+ 12.0.1, Umetrics, Umea, Sweden) (2008) was performed on genus data to identify the effects of crop rotation and management on the bacterial community. The PLS-DA is a particular case of partial least square regression analysis in which Y is a set of binary (0 versus 1) variables describing the categories of a categorical variable on X. In this case, X variables were bacterial genera and binary Y was observations of organic (1) versus conventional (0), or Grain-Only (1) versus Forage-Grain (0) treatments. For this analysis, data were scaled using Unit Variance in SIMCA-P+ (2008). Cross-validation was then performed to determine the number of significant PLS components and a permutation testing was conducted to validate the model. To avoid over parameterization of the model, variable influence on projection value (VIP) was estimated for each genus and genera with VIP < 0.35 were removed from the final model (Pérez-Enciso and Tenehaus 2003; Verhulst et al 2011). R^2X and R^2Y estimates were then used to evaluate the goodness of fit and Q^2 estimate was used to evaluate the predictive value of the model. Scatter- and score-plots were generated only for treatments that were significantly differentiated by the model. The

PLS regression coefficients were used to identify genera that were most characteristic of each treatment group. The positive or negative correlations were considered significant when there was no overlap between the genus 95% confidence interval and the horizontal axis in the PLS regression coefficients graph.

Redundancy analysis (RDA) was carried out using canonical community ordination (CANOCO; Plant Research International BV, Wageningen, The Netherlands) to examine the relationship between abundant phyla and environment variables. Spearman's rank correlations were used to correlate abundant phyla and soil properties using SAS (2008).

5.4 Results

5.4.1 Cropping systems and edaphic soil properties

The total soil C, N, and C: N ratio did not vary significantly under different cropping systems, while pH, organic matter, carbonate C and Olsen P were affected to varying degrees by cropping systems. Total soil C was 3.0 g/kg, 3.2 g/kg 3.1g/kg and 3.0 g/kg under GO, GC, FO, and FC farming systems, respectively. Total soil N was 2.7 g/kg in all of four treatments. C: N ratio was 11.3 under GO and FO farming systems, while it was 11.5 and 10.9 under GC and FC farming systems, respectively. In contrast, pH was 7.0 under organic management systems, higher than that of conventional systems with a pH of 6.7 (P = 0.023). Organic matter was 7.9 under Forage-Grain rotation systems, significantly higher than that of Grain-only rotation systems with 6.7 and 7.2 (P = 0.005). Both rotation and management affected carbonate C and Olsen P (Table 5.1). Carbonate C and Olsen P were much lower under Forage-grain rotation, compared to Grain-only system. Significant correlations existed between total N, C, carbonate C, organic matter and Olsen P, as well as between organic matter, carbonate C, total C and total N (Table 5.2).

5.4.2 Bacterial α-diversity

Bacterial diversity and richness in individual samples under different treatments were calculated (Table 5.3). Statistical differences in richness and diversity were only observed for coverage and ACE at the management level. Percentage of coverage for conventional treatment was higher than that of organic treatment (P = 0.04). The GC had the highest percentage of coverage (84.5%), followed by FC (80.3%), GO (78.8%), and FO (73.2%). The ACE richness was highest for FO (6,147.9), and lowest for GC (3,044.2). The rarefaction curve (Figure 5.1) generated with mothur demonstrated that observed numbers of OTU of FO and GO groups were higher than that of FC and GC groups, with FO having the highest number of observed OTU.

5.4.3 Bacterial community composition

A total of 14 bacterial phyla were found in all the samples, of which seven were abundant (> 1%) (Table5.4). Ninety six percent of soil bacterial sequences belonged to these abundant phyla including*:* *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Gemmatinomadetes*, *Chloroflexi*, *Bacteroidetes* and *Planctomycetes*. *Firmicutes*, *Fibrobacteres*, *Nitrospirae*, *Verrucomicrobia*, *P10*, *TM7* and *WS3* were in low abundance. The phylum distribution fluctuated under different farming disturbances. *Proteobacteria* accounted for 44.5% of total bacterial communities under the GO system, while it was only 27.3% under FC. In contrast, *Actinobacteria* made up 43.1% of total bacterial communities under the FC system, but were present in lower percentage (32.5%) under GO. Phylum *Chloroflexi* was also significantly influenced by management, with the highest percentage (6.8%) found in FC compared to the lowest (3.5%) in GO. A significant interaction

between rotation and system was observed for *Gemmatinomadetes*, *Fibrobacteres*, *Verrucomicrobia*, and *P10*. Percentage of *Nitrospirae* was higher under Forage-Grain farming system. Other phyla did not show significant differences under different treatments. Unassigned bacterial sequences at the phylum level were approximately 1% of the total. In total, eight out of 14 phyla showed significant differences under different farming systems.

The relative abundance of different genera showing significant difference under different treatments was listed in Table 5.5. In phylum *Actinobacteria*, several putative genera including *Blastococcus*, *Lapillicoccus*, *Microlunatus, Pseudonocardia*, *Solirubrobacter*, and *Rubrobacter* showed significant differences among the treatments. The relative abundance of these genera was highest in the FC farming system, followed by GC, FO, and GO. The percentage of different class and genera belonging to the phylum *Proteobacteria* was higher under organic farming system compared to the conventional farming conditions with the exception of *Skermanella*, which was 2.6% and 1.5% under FC and GC farming systems, respectively. Classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were higher in the Grain-Only organic farming system, although the difference was not statistically significant. Class *Deltaproteobacteria* showed the opposite pattern, being highest in Forage-Grain conventional systems, and lowest in Grain-Only organic system. *Pseudomonas* was the predominant genus in *Gammaproteobacteria* with 4.3% in GO, 3.9% in GC, 1.7% in FO, and 0.5% in FC. Within Phylum *Chloroflexi*, genus *Roseiflexus* was significantly influenced by interaction of rotation and management. Other genera in the phyla of *Actinobacteria* and *Proteobacteria* did not show statistical variation among the treatments (Table 5.6 and Table 5.7). There

was no significant fluctuation under different farming systems in other genera within *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, and *Planctomycetes* (Table 5.8).

The PLS-DA analysis showed that there is a significant difference in the composition of bacterial genera between organic and conventional managements (R^2X=0.427, R^2Y=0.882, Q^2=0.159) (Figure 5.2). However, crop rotation (Forage-Grain versus Grain-Only) was not a discriminator factor. Genera that were the most characteristic of each management system were identified using a scatter-plot (Figure 5.3). Among the putative bacterial genera included in the model, *Blastococcus* spp. *Microlunatus* spp. *Pseudonocardia* spp. and *Solirubrobacter* spp. were significantly correlated with conventional treatment, whereas *Gemmatimonas* spp. and *Stenotrophomonas* spp. were highly associated with organic management (Figure 5.3 and Figure 5.4).

5.4.4 Effect of soil edaphic properties on abundant phyla

Canonical correspondence analysis tested the effect of soil edaphic properties on samples and bacterial populations by using an unconstrained analysis (RDA) (Figure 5.5). pH explained 24% of the variance (P = 0.06), CaCO3 C explained 19% (P = 0.02), and the C: N ratio accounted for less than 5% of the variance (P = 0.52). Other soil edaphic variables were highly correlated with each other and were not able to explain variance separately. We also used Spearman's rank order correlation to evaluate relationships between abundant phyla and soil edaphic properties (Table 5.9). It was found that the relative abundance of *Proteobacteria* phylum and *Betaproteobacteria* class was positively correlated with soil pH, while the abundance of *Actinobacteria* was negatively correlated with soil pH. Other phyla did not show significant correlations with soil edaphic properties.

Table 5.1 Physicochemical characteristics of Glenlea soil (0-15cm) on the different treatments (Welsh, 2009; Bell 2012)

Rotation	Management	Total C (g/kg)	Carbonate C (g/kg)	Organic matter (%)	Total N (g/kg)	Olsen P (mg/kg)	pH	C: N Ratio
Grain-Only	Organic	3.0	2.8	6.7	2.6	23.4	7.0	11.3
Grain-Only	Conventional	3.2	2.6	7.2	2.7	21.7	6.6	11.5
Forage-Grain	Organic	3.1	0.4	7.9	2.7	3.6	7.0	11.3
Forage-Grain	Conventional	3.0	1.7	7.9	2.7	14.2	6.6	10.9
SEM		0.06	0.19	0.50	0.008	1.63	0.32	0.19
P-value								
Rotation		NS	<0.0001	0.005	NS	<0.0001	NS	NS
Management		NS	0.0039	NS	NS	0.0035	0.023	NS
Management × Rotation		0.01	0.0001	NS	NS	0.0001	NS	NS

NS= not significant ($P > 0.05$).

Table 5.2 Pearson correlation coefficients between soil edaphic factors[1]

Variables	pH	Olsen P	Total N	Total C	Carbonate C	Organic matter	C: N ratio
pH	1.000	-0.380	-0.414	-0.429	-0.527	-0.424	-0.205
Olsen P		1.000	**0.996**	**0.993**	**0.615**	**0.994**	0.256
Total N			1.000	**0.998**	**0.606**	**0.998**	0.258
Total C				1.000	**0.622**	**1.000**	0.297
Carbonate C					1.000	**0.611**	0.135
Organic matter						1.000	0.297
C: N ratio							1.000

[1]Significant correlations between edaphic factors are indicated in bold type when $P < 0.05$.

Table 5.3 Summary statistics of pyrosequencing 16S rRNA sequences of soil samples

Rotation	Management	Number of trimmed sequences	Mean (SEM) results for indicated variable[1]						
					Richness[3]		Diversity[4]		
			OTU[2] (95% distance)	Coverage (%)	Chao1	ACE	Shannon	Simpson	Effective number of species
Grain-Only	Organic	30,482	1,917	78.8[a,b]	3,660.0	4,936.4[a,b]	7.2	0.0012	562.8
	Conventional	20,473	1,628	84.5[a]	2,889.7	3,044.2[b]	6.8	0.0022	504.4
Forage-Grain	Organic	23,923	2,118	73.2[b]	4,308.7	6,147.9[a]	7.6	0.0005	681.3
	Conventional	31,552	1,860	80.3[a,b]	3,494.4	4,533.6[a,b]	7.2	0.0012	592.2
SEM			236.5	2.7	512.1	677.1	0.22	0.01	122.1
P-value									
Rotation			0.35	0.11	0.20	0.08	0.20	0.38	0.43
Management			0.30	0.04	0.13	0.03	0.20	0.41	0.57
Rotation × Management			0.95	0.83	0.96	0.83	0.97	0.86	0.91

[a, b, c] Means with different letters are significantly different for management at $P < 0.05$.
[1]Mean are from statistical models based on 5 to 6 replicate samples.
[2] OTU= operational taxonomic units.
[3] Based on Chao1 and abundance based coverage estimation (ACE) richness indices.
[4] Based on Shannon and Simpson diversity estimators.

Table 5.4 Phylogenetic composition of bacterial phyla from pyrosequenced 16S rRNA sequences

Phylum	Rotation (Grain-Only)		Rotation (Forage-Grain)		SEM	*P*-value		
	Management		Management			Rotation	Management	Rotation × Management
	Organic	Conventional	Organic	Conventional				
	----------Abundant phyla[1]----------							
Proteobacteria	44.5[a]	32.2[a,b]	34.1[a,b]	27.3[b]	3.13	0.10	0.05	0.70
Actinobacteria	32.5[a,b]	39.2[a,b]	28.4[b]	43.1[a]	2.21	0.97	0.0002	0.08
Acidobacteria	8.5	10.3	13.8	12.1	2.35	0.15	0.88	0.44
Gemmatinomadetes	3.5[B]	3.6[B]	8.6[A]	2.9[B]	1.16	0.15	0.06	0.04
Chloroflexi	3.4[b]	6.1[a]	5.2[a,b]	6.8[a]	0.98	0.18	0.04	0.42
Bacteroidetes	3.3	2.7	2.7	2.2	0.98	0.54	0.52	0.99
Planctomycetes	1.5	1.7	2.1	1.8	0.35	0.30	0.81	0.49
	----------Low-abundance phyla[2]----------							
Firmicutes	0.6	0.9	0.2	1.1	0.34	0.47	0.11	0.32
Fibrobacteres	0.1[B]	0.2[B]	0.3[A]	0.1[B]	0.04	0.05	0.07	0.01
Nitrospirae	0.2[b]	0.2[a,b]	0.3[a,b]	0.5[a]	0.07	0.01	0.21	0.35
Verrucomicrobia	0.2[C]	0.7[A]	0.8[A]	0.5[B]	0.14	0.09	0.42	0.006
OP10	0.2[B]	0.4[A,B]	0.5[A]	0.2[B]	0.08	0.44	0.36	0.04
TM7	0.1	*	0.1	*	0.13	0.98	0.62	0.86
WS3	*	0.2	0.2	0.1	0.15	0.59	0.85	0.56
Unclassified	1.1	1.3	2.1	1.3	0.52	0.45	0.66	0.40

[a,b,c] Means for main effects (rotation or management) are significantly different at $P < 0.05$.

[A, B, C] Means for the interaction between rotation and system are significantly different at $P < 0.05$.

[1]Percentage of sequences larger than 1.

[2]Percentage of sequences smaller than 1.

*Percentage of sequences below 0.1.

Table 5.5 Phylogenetic composition of bacterial phyla from pyrosequenced 16S rRNA sequences

Phylum	Rotation (Grain-only)		Rotation (Forage-grain)		SEM	*P*-value		
	Management					Rotation	Management	Rotation × Management
	Organic	Conventional	Organic	Conventional				
	----------Abundant phyla[1]----------							
Proteobacteria	44.5^{a}	32.2^{a,b}	34.1^{a,b}	27.3^{b}	3.13	0.10	0.05	0.70
Actinobacteria	32.5^{a,b}	39.2^{a,b}	28.4^{b}	43.1^{a}	2.21	0.97	0.0002	0.08
Acidobacteria	8.5	10.3	13.8	12.1	2.35	0.15	0.88	0.44
Gemmatinomadete	3.5^{B}	3.6^{B}	8.6^{A}	2.9^{B}	1.16	0.15	0.06	0.04
Chloroflexi	3.4^{b}	6.1^{a}	5.2^{a,b}	6.8^{a}	0.98	0.18	0.04	0.42
Bacteroidetes	3.3	2.7	2.7	2.2	0.98	0.54	0.52	0.99
Planctomycetes	1.5	1.7	2.1	1.8	0.35	0.30	0.81	0.49
	----------Low-abundance phyla[2]----------							
Firmicutes	0.6	0.9	0.2	1.1	0.34	0.47	0.11	0.32
Fibrobacteres	0.1^{B}	0.2^{B}	0.3^{A}	0.1^{B}	0.04	0.05	0.07	0.01
Nitrospirae	0.2^{b}	0.2^{a,b}	0.3^{a,b}	0.5^{a}	0.07	0.01	0.21	0.35
Verrucomicrobia	0.2^{C}	0.7^{A}	0.8^{A}	0.5^{B}	0.14	0.09	0.42	0.006
OP10	0.2^{B}	0.4^{A,B}	0.5^{A}	0.2^{B}	0.08	0.44	0.36	0.04
TM7	0.1	*	0.1	*	0.13	0.98	0.62	0.86
WS3	*	0.2	0.2	0.1	0.15	0.59	0.85	0.56
Unclassified	1.1	1.3	2.1	1.3	0.52	0.45	0.66	0.40

^{a,b,c} Means for main effects (rotation or management) are significantly different at $P < 0.05$.
$^{A, B, C}$ Means for the interaction between rotation and system are significantly different at $P < 0.05$.
[1]Percentage of sequences larger than 1.
[2]Percentage of sequences smaller than 1.
*Percentage of sequences below 0.1.

Table 5.6 Phylogenetic composition of putative bacterial genera in *Actinobacteria* phylum determined using 16S rRNA pyrosequencing

Family; Genus	Rotation (Grain-Only)		Rotation (Forage-Grain)		SEM	*P*-value		
	Management					Rotation	Management	Rotation × Management
	Organic	Conventional	Organic	Conventional				
	---------- Phylum, ***Actinobacteria*** ----------							
Mycobacteriaceae; *Mycobacterium*	0.3	0.5	0.2	0.2	0.26	0.48	0.76	0.91
Geodermatophilaceae; *Blastococcus*	0.8[b]	1.7[a]	1.2[a,b]	1.7[a]	0.19	0.27	0.002	0.23
Kineosporiaceae; *Kineosporia*	0.5	0.3	0.5	0.2	0.25	0.61	0.31	0.72
Intrasporangiaceae; *Lapillicoccus*	0.3[b]	0.4[a,b]	0.2[b]	0.6[a]	0.09	0.36	0.01	0.11
Microbacteriaceae; *Microbacterium*	1.2	0.4	0.3	0.1	0.30	0.16	0.26	0.91
Micrococcaceae; *Arthrobacter*	2.6	4.6	2.5	3.6	0.97	0.59	0.10	0.76
Micromonosporaceae; *Actinoplanes*	0.8	0.7	1.1	0.6	0.38	0.90	0.47	0.54
Micromonosporacea; unclassified	1.2	1.4	1.5	1.8	0.18	0.08	0.17	0.67
Nocardioidaceae; *Marmoricola*	0.8	0.8	0.6	0.4	0.35	0.44	0.81	0.81
Nocardioidaceae; *Nocardioides*	1.1	1.3	0.6	0.7	0.41	0.24	0.71	0.92
Propionibacteriaceae; *Microlunatus*	1.1[a,b]	2.0[a]	0.8[b]	2.1[a]	0.31	0.78	0.004	0.66
Pseudonocardiaceae; *Pseudonocardia*	0.9[b]	1.7[a,b]	1.3[b]	2.6[a]	0.24	0.02	0.001	0.31
Pseudonocardiaceae; unclassified	0.3	0.4	0.3	0.5	0.07	0.34	0.05	0.23
Solirubrobacteriaceae; *Solirubrobacter*	0.6[b]	1.7[a]	0.8[b]	1.6[a]	0.29	0.88	0.003	0.48
Streptomycetaceae; *Streptomyces*	0.1	0.6	0.3	0.5	0.33	0.34	0.92	0.42
Rubrobacteriaceae; *Rubrobacter*	0.5[b]	1.0[a]	0.3[b]	1.4[a]	0.24	0.57	0.005	0.30
Unclassified Actinobacteria	11.9[a,b]	15.6[a,b]	9.9[b]	17.4[a]	1.81	0.97	0.01	0.33

[a,b,c] Means for main effects (rotation or management) are significantly different at $P < 0.05$.
[A, B, C] Means for the interaction between rotation and system are significantly different at $P < 0.05$.

Table 5.7 Phylogenetic composition of putative bacterial genera in *Proteobacteria* phylum determined using 16S rRNA pyrosequencing

Class; Family; Genus	Rotation (Grain-Only)		Rotation (Forage-Grain)		SEM	*P*-value		
	Management		Management			Rotation	Management	Rotation × Management
	Organic	Conventional	Organic	Conventional				
	----------Phylum, ***Proteobacteria*** ----------							
Alphaproteobacteria	20.5	15.9	18.2	15.0	2.39	0.55	0.15	0.85
Caulobacteraceae; *Brevundimonas*	1.7[a]	0.1[b]	0.2[b]	0.01[b]	0.11	0.15	0.03	0.78
Caulobacteraceae; *Phenylobacterium*	0.5	0.3	0.7	0.3	0.28	0.83	0.42	0.80
Bradyrhizobiaceae; *Balneimonas*	0.4	0.6	0.6	0.6	0.31	0.74	0.72	0.68
Bradyrhizobiaceae; *Bradyrhizobium*	0.5	0.8	0.9	0.9	0.16	0.13	0.41	0.29
Bradyrhizobiaceae; unclassified	0.7	0.6	0.9	0.7	0.28	0.76	0.62	1.00
Hyphomicrobiaceae; *Devosia*	0.6	0.4	0.6	0.9	0.27	0.36	0.24	0.41
Shinella_genera_incertae_sedis; unclassified	0.6	0.4	0.3	0.1	0.23	0.21	0.42	0.64
Xanthobacteraceae; uncultured	0.5[b]	0.8[a,b]	0.7[a,b]	1.1[a]	0.15	0.10	0.05	0.64
Acetobacteraceae; unclassified	0.7	0.7	1.0	0.8	0.39	0.70	0.90	0.76
DA111; unclassified	0.2	0.6	0.4	0.7	0.14	0.61	0.23	0.72
Rhodospirillaceae; *Skermanella*	0.8[b]	1.5[a,b]	1.3[ab]	2.6[a]	0.30	0.02	0.005	0.36
Wr0007; unclassified	0.4	0.5	1.2	0.8	0.37	0.22	0.83	0.55
Erythrobacteraceae; *Altererythrobacter*	0.5	0.3	0.2	0.1	0.22	0.31	0.65	0.99
Betaproteobacteria	10.3	6.3	8.7	5.1	2.00	0.42	0.04	0.93
Alcaligenaceae; *Achromobacter*	0.6	0.1	0.0	0.1	0.19	0.39	0.88	0.47
Burkholderiaceae; *Burkholderia*	0.6	0.0	0.1	0.0	0.19	0.29	0.14	0.99
Comamonadaceae; *Methylibium*	1.0	0.9	1.6	0.9	0.47	0.54	0.44	0.62
Comamonadaceae; Variovorax	1.0	0.4	0.8	0.4	0.34	0.70	0.19	0.94
Oxalobacteraceae; *Duganella*	0.6	0.7	0.3	0.3	0.28	0.31	0.99	0.74
Oxalobacteraceae; *Massilia*	0.8	0.4	0.3	0.1	0.27	0.17	0.25	0.72
Oxalobacteraceae; unclassified	0.4	0.7	0.9	0.5	0.18	0.46	0.93	0.08
Nitrosomonadaceae; uncultured	0.2	0.2	0.6	0.2	0.10	0.11	0.17	0.16
Gammaproteobacteria	11.4	7.1	7.7	3.6	2.72	0.13	0.08	0.67
Enterobacteriaceae; *Pantoea*	0.5	0.2	0.0	0.0	0.17	0.20	0.73	0.98

Pseudomonadaceae; *Pseudomonas*	4.3	3.9	1.7	0.5	2.02	0.10	0.52	0.44
Sinobacteraceae; unclassified	1.4	0.9	1.0	1.1	0.47	0.85	0.69	0.61
Xanthomonadaceae; *Arenimonas*	1.5	0.4	1.0	0.6	0.41	0.94	0.12	0.47
Xanthomonadaceae; *Pseudoxanthomonas*	0.1	0.0	0.6	0.0	0.18	0.56	0.24	0.62
Xanthomonadaceae; *Stenotrophomonas*	0.7[a]	0.0[b]	0.3[a]	0.0[b]	0.25	0.67	0.04	0.71
Xanthomonadaceae; *Thermomonas*	0.6	0.2	0.2	0.1	0.21	0.44	0.41	0.75
Xanthomonadaceae; *Xanthomonas*	0.5	0.1	0.2	0.0	0.18	0.47	0.33	0.87
Xanthomonadaceae; unclassified	0.7	0.5	1.1	0.5	0.36	0.69	0.18	0.45
Deltaproteobacteria	1.0[b]	2.7[a,b]	3.4[a]	3.5[a]	0.05	0.009	0.10	0.13
Unclassified Proteobacteria	3.9	4.5	6.1	4.4	0.58	0.03	0.26	0.03

[a,b,c] Means for main effects (rotation or management) are significantly different at $P < 0.05$.
[A, B, C] Means for the interaction between rotation and system are significantly different at $P < 0.05$.

Table 5.8 Phylogenetic composition of bacterial genera in Acidobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, Planctomycetes generated using pyrosequenced 16S rRNA sequences

Family; Genus	Rotation (Grain-only)		Rotation (Forage-grain)		SEM	*P*-value		
	Management					Rotation	Management	Rotation × Management
	Organic	Conventional	Organic	Conventional				
	----------Phylum, Acidobacteria ----------							
Acidobacteriaceae; *Chloroacidobacterium*	0.4	0.6	0.9	0.5	0.33	0.51	0.97	0.41
Acidobacteriaceae; uncultured	7.1	8.6	8.3	11.0	0.26	0.46	0.40	0.86
Acidobacteria; unclassified	1.1	1.7	4.2	2.2	0.85	0.06	0.76	0.18
	----------Phylum, Bacteroidetes ----------							
Flavobacteriaceae; *Flavobacterium*	0.5	0.8	1.5	0.4	0.45	0.77	0.38	0.12
Chitinophagaceae; unclassified	0.6	0.7	1.0	0.5	0.36	0.97	0.52	0.38
Cytophagaceae; *Hymenobacter*	0.6	0.1	0.1	0.0	0.18	0.19	0.41	0.76
	----------Phylum, Chloroflexi ----------							
Anaerolineaceae; unclassified	0.6	1.2	0.9	1.2	0.41	0.63	0.25	0.71
Chloroflexaceae; *Roseiflexus*	0.6	1.5	1.1	2.3	0.28	0.02	0.002	0.52
Unclassified	0.4	0.6	0.7	0.5	0.32	0.76	0.97	0.52
	----------Phylum, Firmicutes ----------							
Bacillaceae; *Bacillus*	0.4	0.5	0.2	0.4	0.25	0.42	0.45	0.66
	----------Phylum, Gemmatimonadetes ----------							
Gemmatimonadaceae; *Gemmatimonas*	1.9	1.1	2.7	1.8	0.73	0.29	0.23	0.87
Gemmatimonadaceae; unclassified	1.8	2.7	4.1	3.9	1.16	0.11	0.63	0.53
Unclassified	0.1	0.2	0.4	0.5	0.23	0.27	0.50	0.78
	----------Phylum, Planctomycetes ----------							
Unclassified	0.9	0.9	1.3	0.9	0.27	0.59	0.51	0.48

Table 5.9 Spearman's rank correlations between abundant phyla with soil properties[1]

Abundant phyla	Correlation						
	pH	Olsen P	Total N	Total C	Carbonate C	Organic matter	C: N ratio
Proteobacteria	**0.61**	0.07	-0.04	-0.03	-0.06	-0.03	0.05
Alphaproteobacteria	0.46	-0.47	-0.09	-0.12	-0.17	-0.11	-0.17
Betaproteobacteria	**0.62**	-0.18	-0.24	-0.38	-0.36	-0.20	-0.36
Gammaproteobacteria	0.25	-0.004	-0.05	-0.15	-0.13	0.04	-0.13
Deltaproteobacteria	-0.47	0.18	-0.21	-0.15	-0.13	-0.04	-0.13
Actinobacteria	**-0.65**	0.44	0.51	0.50	0.41	0.50	-0.17
Bacteroidetes	0.33	-0.37	-0.37	-0.36	-0.53	-0.36	0.38
Chloroflexi	-0.53	0.33	0.40	0.39	0.40	0.39	0.09
Firmicutes	-0.21	0.47	0.53	0.52	0.33	0.52	0.35
Gemmatimonadetes	-0.08	-0.46	-0.45	-0.45	-0.30	-0.45	-0.03
Planctomycetes	-0.18	-0.28	-0.21	-0.24	-0.31	-0.24	-0.16
Acidobacteria	-0.06	-0.23	-0.16	-0.20	-0.35	-0.20	-0.15
Nitrospirae	-0.004	-0.24	-0.19	-0.27	-0.40	-0.27	-0.23
Unclassified	-0.39	-0.47	-0.41	-0.41	-0.19	-0.41	-0.14

[1]Significant correlations between edaphic factors are indicated in bold type when $P < 0.05$.

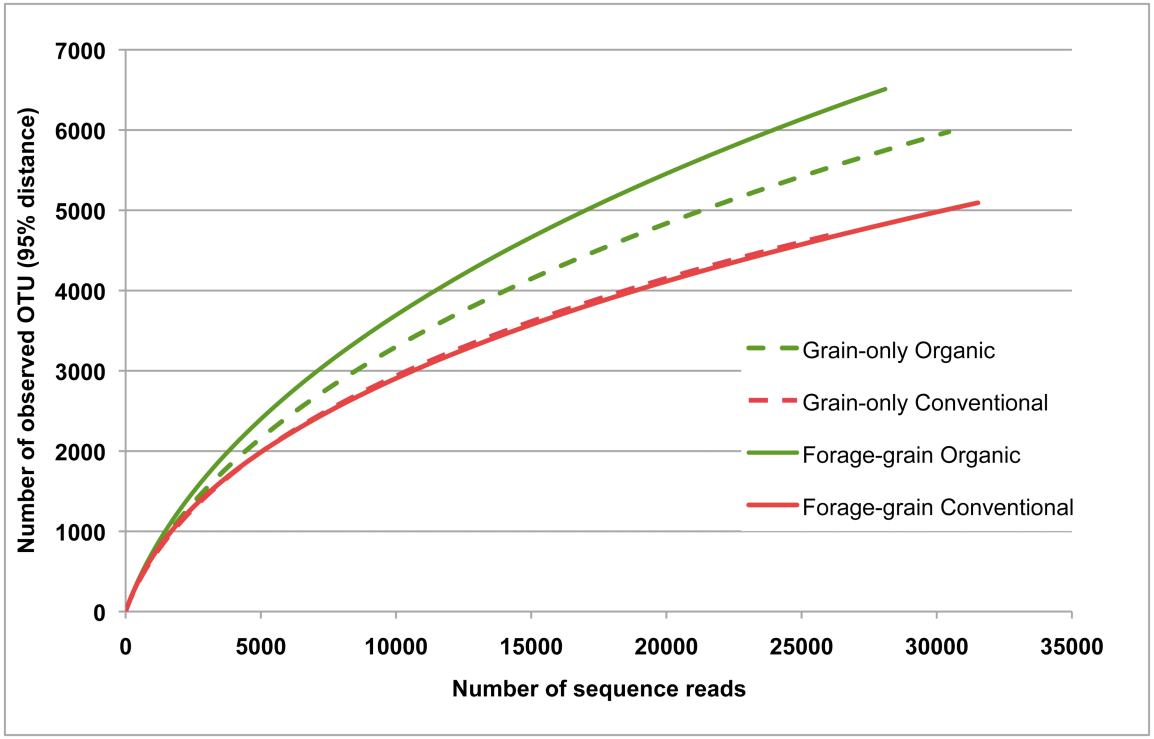

Figure 5.1 Rarefaction curves for pooled samples within each treatment at OTU cutoff of 0.05 distance.

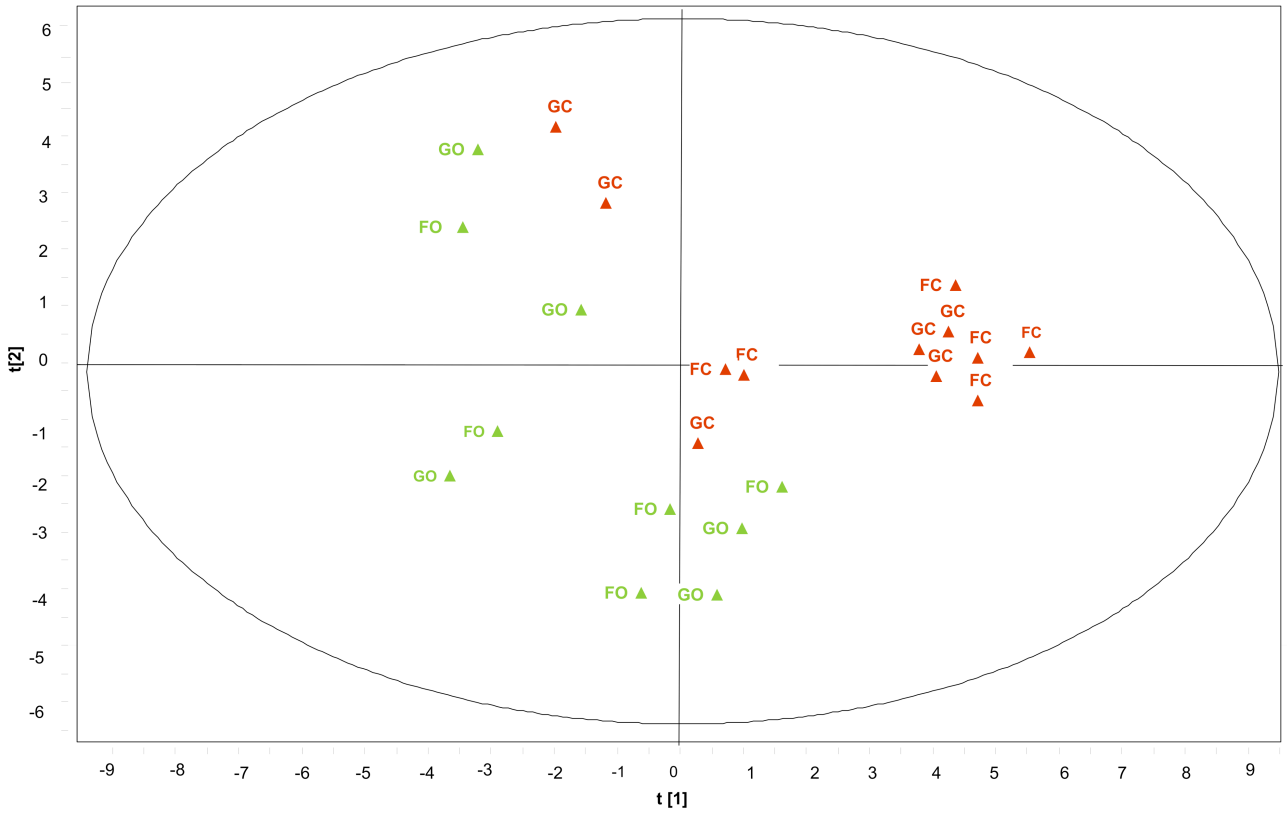

Figure 5.2 Partial least square discriminant score plot of soil bacteria under organic and conventional treatments. GO: Grain-Only organic; GC: Grain-Only conventional; FO: Forage-Grain organic; FC: Forage-Grain conventional. Model indicated a significant difference in the composition of putative bacterial genera between organic and conventional managements (R^2X=0.427, R^2Y=0.882, Q^2=0.159). Only genera with VIP > 0.35 is included in the model.

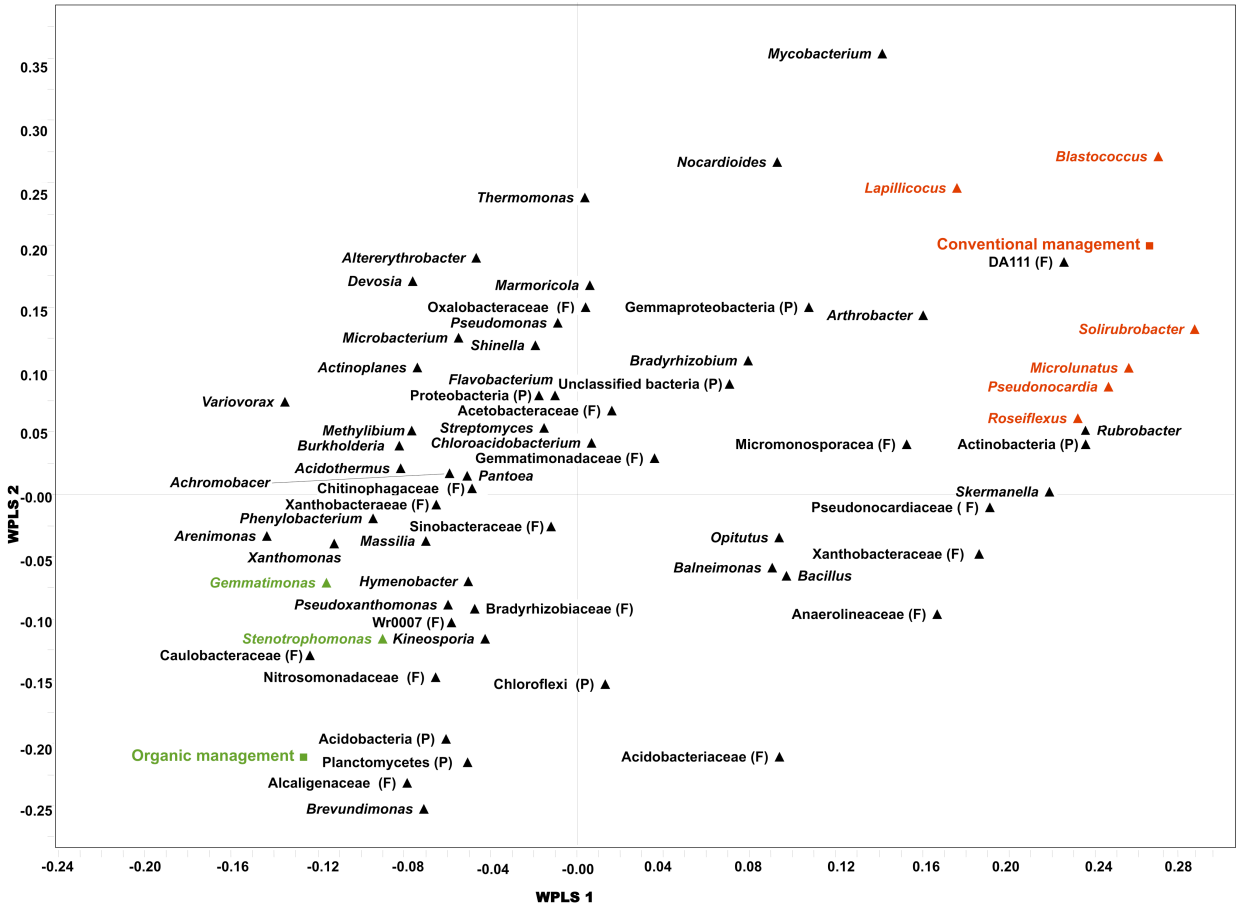

Figure5.3 Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundance of the putative bacterial genera in soil microbiome and their association with organic or conventional treatments. Bacterial genera closer to organic or conventional are highly correlated to either treatment. PLS1 (R^2X=0.27, R^2Y=0.525, Q^2=0.186) and PLS2 (R^2X=0.127, R^2Y=0.218, Q^2=-0.081). Some sequences could only be affiliated to phylum (P) or family (F) levels.

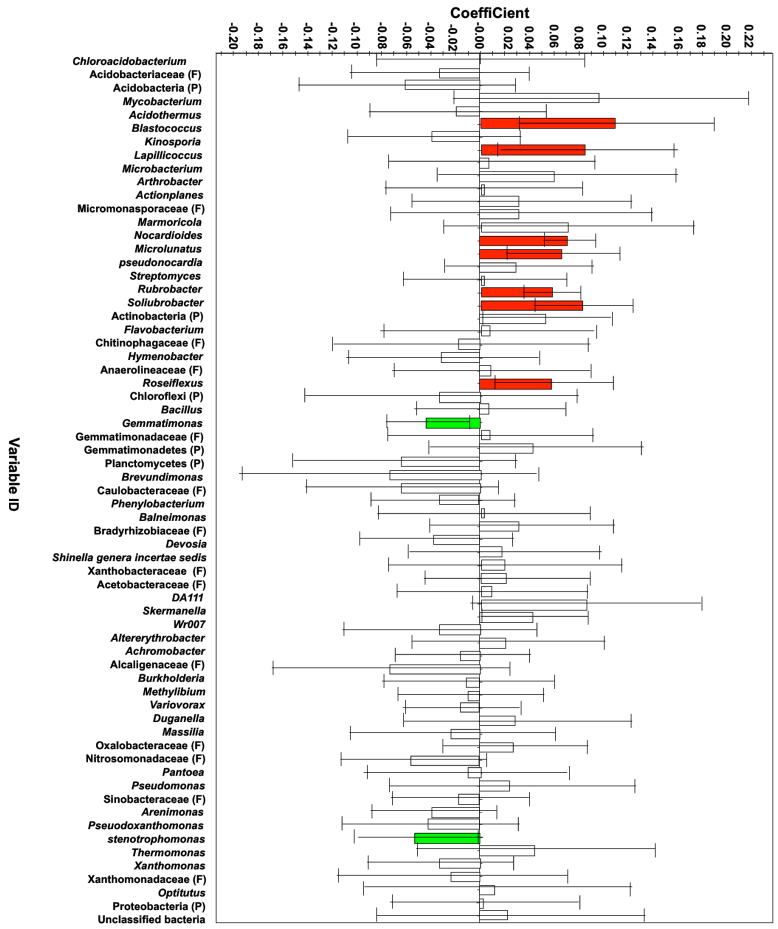

Figure 5.4 Coefficient plot of the bacterial profiles of organic and conventional treatments. Partial least squares discriminant analysis (PLS-DA) coefficient plot based on the relative abundant of the bacterial genera in the microbiome profile of organic and conventional treatments. Genera with significantly positive (>0) or negative (<0) PLS regression coefficients (i.e. no overlap between the 95% confidence interval indicated and the horizontal axis) contribute significantly to the prediction of the organic (green bars) or conventional samples (red bars).

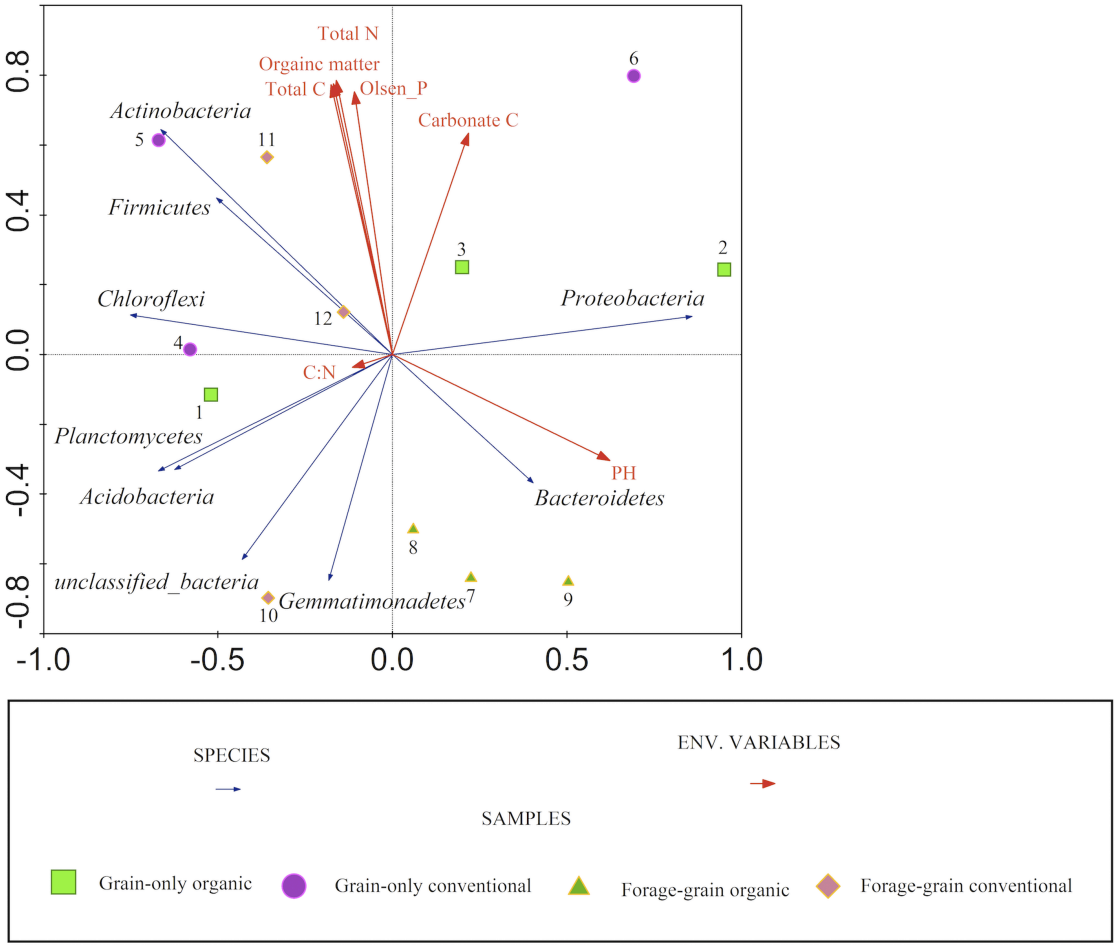

Figure 5.5 Redundancy analysis ordination plots of abundant phyla for individual sample of different cropping managements.

5.5 Discussion

In this study, crop rotation and management strategies did not alter total C, total N and C: N ratio but significantly affected organic matter, soil pH, carbonate C and Olsen P (Table 5.1). This indicates that farming systems gradually but not dramatically change soil edaphic properties (Vandiepeningen et al 2006). We observed that organic management led to a neutral soil pH compared to conventional practices (7.0 versus 6.6; Table 5.1). This might be due to the application of synthetic fertilizer that could acidify the soil in the conventional systems (Barak et al 1997). Other reports indicated that soil pH could be influenced by other soil traits such as C:N ratio (Kuramae et al 2012), vegetation, or soil type (Ziadi and Sen Tran 2008). However, in our study, the field trials were run under identical conditions, and the soil pH was not significantly correlated with other soil edaphic characteristics (Table 5.2). Therefore, the farming system was the sole factor to change the soil pH. As we expected, soil organic matter was higher under Forage-Grain rotations (Table 5.1) (Su 2007; Kuramae et al 2012). However, organic farming system did not increase organic matter in the soil surface, compared to conventional farming system. In contrast, other studies have shown that organic matter was higher in the top 0.3m of soil under organic management (Mader et al 2002; Pimentel et al 2005). This discrepancy could be due to different crops contributing to different amount of biomass and no additional manure added to our trials (Bell et al 2012).

We used high-resolution power of 454-pyrosequencing to obtain insight into the effects of farming management styles (organic, conventional) and crop rotations (Grain-Only, Forage-Grain) on the diversity, richness and composition of soil bacterial

communities. In total, pyrosequencing identified 14 phyla and 178 putative genera of bacteria in different soil samples. We found that organic and conventional farming management had major influence on soil bacterial communities while the effects of crop rotation were of smaller magnitude. We were also able to identify putative genera that were correlated with either organic or conventional farming management.

It has been reported that organic farming systems enhance microbial diversity in soil compared to the conventional systems (Mader et al 2002). Although not statistically significant, we found a similar trend in this study (Table 5.3). Previous research indicated that soil pH might be the primary factor influencing richness and diversity of bacterial communities (Ramirez et al 2010) with the highest richness and diversity found to be near the neutral pH. Lauber et al (2009) proposed that bacterial diversity had a strong negative relationship with soil pH when it was lower than 6.5. In this study, soil pH ranged from 6.6 to 7.0, and was significantly higher for organic compared to conventional system. This indirectly indicates that organic management might favor higher bacterial diversity. And it is important to note that standard diversity parameters only based on OTU without taxonomic identity of the different groups is not sensitive enough to detect the influence of agriculture management on the soil bacterial community, because changes in some taxonomic groups might be compensated by changes in others (Hartmann and Widmer 2006). Lending support to this hypothesis, we detected a significant shift in soil bacterial communities due to farming systems when sequences were taxonomically ranked (Figure 5.3).

When sequences were affiliated to taxonomic level, bacterial populations fluctuated under different farming systems. An interesting observation in this study was the greater percentage of phylum *Proteobacteria*, including classes *Alphaproteobacteria*,

Betaproteobacteria and *Gammaproteobacteria* in organic farming management (39.3%) compared to the conventional system (29.7%). To interpret these findings in an ecological context and to explain why some bacterial phyla are more abundant in soil than others, some researchers have used the concept of the copiotrophic versus oligotrophic bacteria (Meyer 1994; Fierer et al 2007). Copiotrophic bacteria (fast growing) flourish in soils with large amounts of available nutrients, while oligotrophic groups (slow growing) predominate in soil having low nutrient availability. It has been proposed that oligotrophic bacteria are more associated with organically than conventionally farmed soils due to low availability of organic carbon and nitrogen (van Bruggen and Semenov 2000; Vandiepeningen et al 2006). Among *Proteobacteria*, *Betaproteobacteria* are considered as copiotrophic (Vandiepeningen et al 2006; Fierer et al 2007), and thus, their population is expected to be lower in organic farming. There is no indication if other classes within *Proteobacteria* can be classified into copiotrophic-oligotrophic scheme (Fierer et al 2007). In our study we found higher *Betaproteobacteria* in organic farmed soil. As the organic farming system did not contribute to a higher amount of top bulk soil total C, total N and organic matter compared to the conventional system, higher relative abundance of these bacteria could be due to other factors. It was found that *Proteobacteria* and *Betaproteobacteria* were highly correlated with pH in this study ($P< 0.05$, Table 5.9); therefore, we assumed that neutral pH could increase the abundance of these bacteria in soil.

We believe that because of enormous phylogenetic and physiological diversity within each bacterial phylum, it is unlikely that an entire phylum demonstrates the same ecological characteristics. An example would be *Burkholderia*, a genus in *Betaproteobacteria* that exhibits oligotrophic traits due to their catabolic versatility that enables them to degrade recalcitrant compounds and survive in environments with limited

nutrient availability (Suárez-Moreno et al 2011). Thus, the hypothesis that oligotrophic bacteria are primarily associated with organically farmed soil could simplify the ecological categories of bacterial communities in soil.

In this study, we found a higher population of *Brevundimonas* spp., *Burkholderia* spp., *Pseudomonas* spp., and *Stenotrophomonas* spp. in organic farming systems (Table 5.7). These genera are ubiquitously found in the soil and several of their species have important ecological roles in nutrient cycling and suppression of plant diseases (Haas and Défago 2005; Ryan et al 2009; Suárez-Moreno et al 2009). For instance, members of *Stenotophomonas, Pseudomonas and Burkholderia* genera can fix nitrogen (Park et al 2005; Suárez-Moreno et al 2009). Higher relative abundance of these genera might help maintaining total N level in the organic farming soil without fertilizer supplementation. In addition, many plant growth-promoting bacteria (PGPB) belong to *Burkholeria*, *Stenotrophomonas* and *Pseudomonas* genera, which were more abundant in organic farming system (Table 5.7). Interestingly, these genera were abundant in soils planted with alfalfa, wheat, oilseed rape and various weeds (Ryan et al 2009; Haas and Défago 2005). Because organic farming systems support more weeds than the conventional farming systems, it might promote these PGPB populations (Entz et al 2004). However, it is important to notice that not all species in these genera are PGPB and there are species, which are pathogenic to humans, animals and plants (i.e. *P. aerugionsa*, *P. syringae*, and *S. maltophilia* K279a*)*. The 16S rRNA marker genes have a limited ability to identify bacteria up to the species level, and thus other methodologies with high resolution including metagenomic shotgun sequencing (Segata et al 2012) must be applied in order to differentiate PGPB from pathogenic species in the soil bacterial community.

The percentage of *Actinobacteria* and *Chloroflexi* were lower in organic (30.4% and 4.3%, respectively) compared to the conventional system (41.1% and 6.4%, respectively). Our results show that the conventional farming system increases the actinobacterial proportion in the community with no change in their composition, compared to the organic farming system. The PLS-DA loading scatter and coefficient plots (Figure 5.3 and Figure 5.4) indicated that the conventional farming system supported a higher population of several genera within *Actinobacteria*, including *Blastococcus* spp., *Microlunatus* spp., *Pseudonocardia* spp. and *Solirubrobacter* spp. *Actinobacteria* which are able to degrade a variety of organic compounds including some herbicides and pesticides (De Schrijver and De Mot 1999). *Pseudonocardia* spp. has been reported to degrade environmental contaminants, particularly aromatic hydrocarbons or compounds that contain aromatic rings (Lee 2004). As such, herbicides and pesticides sprayed containing aromatic rings may have favored bacteria, such as *Pseudonocardia* spp., capable of degrading them. Some *Microlunatus* spp. show high levels of phosphorus accumulation function and phosphate uptake/release activities (Akar et al 2005). Therefore, in a conventional farming system where pesticides and inorganic fertilizers are commonly used to increase the crop yield, high availability of substrate for *Microlunatus* spp. and other actinobacterial species could boost their population.

Actinobacteria also play a major role in turnover of organic matter and carbon cycling. They can decompose some recalcitrant carbon sources including cellulose and chitin (Acostamartinez et al 2008; Jenkins et al 2010). Organically farmed soils have been reported to be rich in recalcitrant carbon sources (Fließbach et al 2007), and the diversity of *Actinobacteria* would be expected to be higher in those soils than in conventionally farmed soils. However, in our organic farming fields the recalcitrant carbon sources were not

higher in the surface soil compared to the conventional farmed fields (Bell et al 2012). Therefore, recalcitrant carbon sources could not drive the increasing diversity of *Actinobacteria* in our study.

A number of studies have shown that soil edaphic factors shape microbial communities (Lauber et al 2008; Ziadi and Sen Tran 2008; Singh et al 2009; Nacke et al 2011). In our study, we found that proportions of abundant phyla were highly affected by soil pH. Our observations were consistent with other studies that demonstrated pH was one of the main drivers of change in soil bacterial communities from continental scale (Lauber et al 2009) to small landscape (Singh et al 2009; Nacke et al 2011). At the phylum level, *Proteobacteria* were positively correlated with soil pH, while *Actinobacteria* were negatively correlated and *Acidobacteria* had a very weak correlation with soil pH. Our results are in contrast to some studies that showed *Actinobacteria* significantly increased with higher pH values, and *Acidobacteria* was dependent on soil pH (Jones et al 2009; Nacke et al 2011). The soil pH value varied significantly from 3 to 8 in other studies, while the soil pH in our experiments only varied from 6.6 to 7 which could be the reason for the lack of change in *Acidobacteria* populations. In our study, *Betaproteobacteria* and *Alphaproteobacteria* populations increased with higher soil pH, while *Deltaproteobacteria* declined. This result was consistent with what was reported by Nacke et al. (Nacke et al 2011). Our studies demonstrated that *Proteobacteria* and *Actinobacteria* were more sensitive to pH variation than other bacterial phyla.

Finally, it is important to acknowledge that the choice of target variable regions of 16S rRNA may have affected the outcome of species richness and diversity analyses because the sequence divergence is not distributed evenly along the 16S rRNA gene (Liu et al 2008; Kim et al 2011). We deep sequenced the V1-V3 regions of the bacterial 16S

rRNA, which covered the V2-V3 region, suitable for distinguishing most bacterial species ranging from the phylum level to the genus level (Chakravorty et al 2007; Kim et al 2011). Therefore, even if some bacterial communities have missed or overestimated, the overall shifting in the phylogenetic composition of bacterial communities under different treatments have been assessed.

Conclusion

We demonstrated that different farming practices significantly changed the relative abundances of *Proteobacteria* and *Actinobacteria*. Farming management practices (organic versus conventional) rather than crop rotation (Grain-Only versus Forage-Grain) appeared to have a strong impact on shifting the abundance of soil bacterial communities, which could translate to changes in soil quality and productivity. Some bacterial groups, such as *Gemmatinomadetes*, *Fibrobacteres*, *Verrucomicrobia* and *OP10* were influenced by the interaction of crop rotation and management. Most soil properties including C: N ratio, total N, total C, Olsen P, and organic matter, did not play a major role in shaping bacterial communities. However, pH had the strongest effect on the bacterial community structure. Organic farming systems led to a neutral pH, which might be beneficial to *Proteobacteria*. On the other hand, conventional farming systems supported a higher percentage of *Actinobacteria*. Therefore, neither organic farming nor conventional farming can address all the aspects of beneficial soil bacterial communities, which is crucial to soil quality and productivity. Further research is required to investigate the shifts in diversity of beneficial bacterial and fungal pathogens under different farming systems in the long run.

6.0 GENERAL DISCUSSION

Maintaining soil health is a basic requirement of sustainable agricultural production, and maintaining the biodiversity of soil microbes is essential to preserving soil health. Bacterial communities in the soil serve fundamental functions in maintaining the health and productivity of the soil and residing plants. These microbes are associated with the processes of soil structure formation, organic matter decomposition; toxin removal and the cycling of C, N and P and other micronutrients. They also help maintain the health of plants through multifaceted mechanisms, such as inducing systemic resistance, aiding nutrient uptake, promoting plant growth, and suppressing pathogens (Ongena et al 2004; Zehnder et al 2001; Weller et al 2002; Suman et al 2005; Basak et al 2010). Reciprocally, the composition and function of bacterial communities is influenced by soil health, which is in turn influenced by different environmental perturbations, most commonly farming activity. However, most recent studies of different farming practices have focused on the biomass, respiration rates, and enzyme activity of bacterial communities, with little attention being paid to the response of bacteria at the community level (Peixoto et al 2006). Furthermore, the results of these studies were not consistent. One explanation for these discrepancies differences in the resolution of the methodologies used to analyze complex bacterial communities in the soil (Wu et al 2007; Joergensen et al 2010; Sugiyama et al 2010). With the use of advanced technologies, such as terminal restriction fragment length polymorphism (TRFLP) and pyrosequencing, it is now possible to study in detail how bacterial communities respond to the stress of different soil perturbations.

Some of the experiments in this study were conducted on Canada's longest running organic and conventional comparison site, the Glenlea Research Station in Manitoba, Canada. The purpose of this study was to assess the influence of different agricultural practices on the composition and function of soil bacteria at the community level. At the

second site, the Ian Morison Research Station (Carman, MB), we evaluated the changes that occur in bacterial communities under varying cropping systems and practices, including monoculture, rotation, and tillage.

This study has resulted in several major findings and was successful in outlining the effect of different soil conditions on bacterial composition and function at the community level. They are as follows:

1. Different cropping systems influence the relative abundance of bacterial communities and plant growth-promoting rhizobacteria (PGPR) groups at both the phylum and genus level.
2. Organic cropping systems were found to be beneficial to bacteria from the phylum Proteobacteria, whereas conventional cropping systems favored bacteria from the phylum Actinobacteria. Organic cropping systems produced neutral soil pH resulting in higher richness and diversity of bacterial communities in the soil.
3. Canola rotation was more beneficial to Proteobacteria when compared to canola monoculture, wheat monoculture, and wheat rotation. Zero tillage supported a higher percentage of Firmicutes. This phylum contains many PGPR.
4. The phylum Proteobacteria contains several genera rich in plant growth-promoting rhizobacteria such as *Pseudomonas*, *Stenotrophomonas, and Brevundimonas*. Organic and farming systems contained a higher percentage of bacteria from these genera.
5. Zero tillage organic farming systems resulted in increased numbers of *Pseudomonas* species that produce antibiotics. In Manitoba soils, bacterial strains producing the antibiotics Phenazine and pyrrolnitrin were prevalent, while 2,4-DAPG and pyoluteorin producing strains were not found.

6. *Pseudomonas margnalis* strains were found to produce phenazine and pyrrolnitrin.

As mentioned previously, earlier studies on the influence of cropping systems on bacterial communities produced inconsistent results, whether they were based on culturing methods or molecular methods. To avoid a similar situation, we applied both selective media culture methods and cutting edge molecular techniques (TRFLP and pyrosequencing) to gain a comprehensive picture of the interaction between bacterial communities and cropping systems. Cropping systems were found to influence not only the structure of bacterial communities but also the production of antibiotics.

Bacterial antagonists are bacteria that suppress the growth of other organisms. Bacterial antagonists identified in this study were influenced by different cropping systems. Compared to conventional farming systems, organic farming supported a higher number of *Pseudomonas* antagonists, as determined using both culture and molecular methods. Organic farming systems supported more weeds than the conventional farming systems, and thus the *Pseudomonas* population was promoted. Pyrosequencing also revealed a larger population of *Brevundimonas* spp., *Burkholderia* spp., and *Stenotrophomonas* spp. in organic farming systems. These bacterial genera have important ecological roles in nutrient cycling and in the suppression of plant diseases (Suárez-Moreno et al 2011; Ryan et al 2009; Haas et al 2005). Pyrosequencing revealed a higher percentage of bacteria from the phyla Actinobacteria and Chloroflexi were present in conventional systems. It has been previously reported that Actinobacteria are able to degrade a variety of organic compounds including herbicides, pesticides, and fertilizers (De Schrijver and De Mot 1999; Lee 2004; Akar et al 2005). Herbicides, pesticides, and fertilizers are widely used in conventional farming systems; this likely promotes a rise in the actinobacterial population.

By combining TRFLP and pyrosequencing, we achieved consistent results in the richness and diversity indices in bulk soil under different tillage practices. A higher richness and diversity was observed in ZT soil, and both TRFLP and pyrosequencing showed that there was a difference in bacterial communities when compared to tilled soil (Fig. 3.8 and Fig. 4.4). The culture based method showed that the ZT soil harbored a significantly higher number of *Pseudomonas* spp. (Table 3.3). Pyrosequencing demonstrated a similar result, even though not statistically significant (Table 4.4). Zero tillage soil was also found to contain a relatively higher percentage of bacteria from the phylum Firmicutes.

Previously, Spedding et al. (2004) used PLFA analysis to report that bacterial communities did not change significantly under different tillage management. Our pyrosequencing data corroborated findings from this study and also showed that the majority of bacterial taxonomic composition in bulk soil did not shift significantly in response to tillage practices. On the other hand, it has been reported that tillage alters many physical and biochemical properties of soil, including bulk density (Wander et al., 1998), aggregation (Chan and Mead 1988), and microbial biomass (Kandeler et al., 1999). However, this increase in the proportion of microbial biomass from reduced tillage to no tillage is attributable to fungi (Bear et al 1997; Frey et al 1999). As such, our results along with the previous study by Spedding et al. (2004) showed that bacterial communities are more resilient to tillage practices.

Both TRFLP and pyrosequencing found that there were significantly different bacterial communities associated with canola and wheat fields. Farming practices (rotation vs. monoculture) also played a role in shaping bacterial communities. The similarity between canola rotation and canola monoculture was lower than that of wheat rotation and

monoculture. As reported previously, arbuscular mycorrhizal fungi (AMF) play a role in shaping bacteria communities (Toljander et al 2007). Since canola is a non-AMF crop, this explains part of the variation in bacterial communities relative to wheat. Additionally, canola root can release S-containing compounds, such as glucosinolates and isothiocyanates, that inhibit the growth of some microorganisms (Brown and Morra 1997; Kirkegaard et al 2001; Rumberger and Marschner 2002; Pascault 2010). When canola is continuously grown, the AMF communities presumably decline and the concentration of S-containing compounds rise. These results in different bacterial consortia compared to canola rotation. Wheat can consistently support AMF communities whether in monoculture or rotation; therefore, bacterial communities were more consistent between the two.

Both TRFLP and pyrosequencing revealed that farm management (organic vs. conventional), rather than rotation (grain_only vs. forage_grain), was the discriminating factor in shaping the bacterial communities in our trials. The choice of organic vs. conventional farming significantly affects the soil pH, which was found to be the main driver in shifting soil bacterial communities from continental scale to small landscape (Lauber et al 2009; Singh et al 2009; Nacke et al 2011). Furthermore, we also found that Actinobacteria and Proteobacteria were more sensitive to pH variation than other bacterial phyla.

It is important to note that the methodologies used in this study have some limitations. Culturing bacteria selects for the fasting growing species at the temperature set in this study. A workaround would be to use lower temperatures to select for slow growing bacteria. For the molecular techniques, the 16S rRNA genes analyzed can often be too similar between species to distinguish different bacteria. To compensate for this, different genes can be chosen, other than 16S rRNA gene, to more accurately determine the

composition and function of bacterial communities in the soil. For example, the *rpoB* gene encoding the RNA polymerase beta subunit can be used to analyze bacterial communities (Peixoto et al 2006). Alternatively, higher resolution methodologies including metagenomics shotgun sequencing can be applied to differentiate plant growth-promoting rhizobacteria from pathogenic species in the soil.

Future research should focus on including fungal communities and pathogen populations to more comprehensively understand the interaction between the entire microbial consortium in the soil and cropping practices. A better understanding of the soil ecosystem would allow us to design cropping practices that maintain the diversity of microbial communities. Consequently, maintaining a healthy productive soil would allow us to achieve a more sustainable agricultural system(s).

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