Microbial diversity of buckwheat rhizosphere in wireworm-infested and non-infested soils using metagenomics

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ABSTRACT

Wireworm has become a major problem causing extensive crop loss in many potato production areas in Canada.Wireworm control methods includes the use of chemical pesticides. However, pesticides can affect human health and the environment, and their use has consequently been questioned and prohibited in many countries. Therefore, the use of environmentally friendly plant protection techniques, including crop rotation as an alternative to chemical control measures have been promoted to minimize crop damage. In that context, buckwheat is used as a rotation crop to mitigate wireworm damage in potatoes. But so far, less is known about how buckwheat contributes to mitigate disease. This study was designed in a context of integrated pest management, with primary objectives to: (1) determine the microbial diversity in the buckwheat rhizosphere in comparison with other rotation crops; and (2) determine the correlation between the buckwheat rhizosphere microbiome structure and wireworm density. To achieve these objectives, 16S rRNA metagenomic sequencing was performed to determine the microbial diversity in bulk and rhizosphere soils of buckwheat and barley grown at two locations during two growing seasons. A pilot wireworm trapping study was also performed to assess the wireworm population following buckwheat and barley as rotation crops. The study identified 27 phyla in the two crops of which *Proteobacteria, Bacteroidetes, Actinobacteria* were the most abundant and species identification was confidently achieved in 7 phyla including *Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes, Firmicutes, Deinococcus-Thermus* and *Crenarchaeota*. Interestingly, *Methylophilus flavus, Saccharopolyspora tripterygii* and *Deinococcus yunweiensis* were three operational taxonomic units (OTUs) found at the species level to be unique to the buckwheat rhizosphere soil at both locations and purported as non-pathogenic entophytic bacteria and beneficial for sustainable agriculture. Moreover, after two years, a reduction in wireworm density was observed in both crops although, the direct link associating the reduced wireworm density and the observed microbial diversity and the operating mechanisms in each crop remain to be elucidated. Taken together, changes were observed in the soil microbial communities associated with specific rotation crops and a reduction in wireworm density was correlatively observed. Thus, our study showed that the root system of buckwheat influences the structure of the microbiome in the rhizosphere as hypothesized.

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List of Abbreviations

AAFC	Agricultural and Agri-food Canada
AMF	Arbuscular Mycorrhizal Fungi
B	Boron
bp	Base pair
C	Carbon
Cu	Copper
Cl	Chloride
CO_2	Carbon dioxide
DAPG	Diacetylphloroglucinol
DNA	Deoxyribonucleic acid
DGGE	Denaturing Gel Gradient Electrophoresis
Fe	Iron
FAO	Food and Agriculture Organization
HR	Hypersensitive Response
IPM	Integrated Pest Management
ISR	Induced Systemic Resistance
K	Potassium
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
N	Nitrogen
P	Phosphorus
PGPR	Plant Growth Promoting Rhizobacteria
PGM	Personal Genome Machine
pH	Potential of Hydrogen
RNA	Ribonucleic acid
SNP	Single Nucleotide Polymorphism
VOCs	Volatile Organic Compounds
Zn	Zinc

CHAPTER ONE: Introduction and Literature Review

1.1 Introduction

Agricultural plant production is essential to humans for their food supply, and feeding growing globe populations has become challenging (Studacher et al., 2013). According to projections of the Food and Agriculture Organization of the United Nations (FAO), the demand for food will increase by 70% by 2050 (Umesha et al., 2017; Barea, 2015) and will double in developing countries. As reported by Ray et al. (2013), the world's demands for crop productivity are increasing due to the growth of the human population, food and non-food uses. Therefore, agricultural production around the world must be increased by 60%–110% to meet the expected demands (Ray et al., 2013). However, increasing yield and sustainable crop production face many challenges including climate change, plant pests, diseases, weeds as well as drought (Ahkami et al., 2017) and flooding (Saussure et al., 2015; Oerke and Dehne, 2004). To increase the yield of basic food crops, additional inputs for production and new technologies are essential and needed for managing crop pests and diseases (Bargaz et al., 2018).

Recently, significant losses in crop value and quality have been attributed to wireworm in many growing areas across the world (Saussure et al., 2015; Furlan, 2005; Barsics et al., 2013; Ansari et al., 2009; Knodel and Shrestha, 2018; Wallinger et al., 2013). According to Saussure et al., (2015), wireworms were reported in the early twentieth century as a pest in Europe (Traugott et al., 2008; Saussure et al., 2015), and they have caused economic losses estimated at 472 million Euros only by attacking maize roots (Studacher et al., 2013; Wesseler and Fall, 2010). The study conducted by Saussure et al. (2015) reported wireworm attacks in 10% of maize plants from germination stage to the eight leaf-stage (Taupin, 2007) or in some situations until the twelve leaf phenological stage. Likewise, in North America and United Kingdom, crop losses caused by wireworms range between 5% - 25% (Saussure et al., 2015).

Wireworms are the soil dwelling larvae of click beetles (Coleoptera: Elateridae) (Furlan, 2005; Barsics et al., 2013; Laznik et al., 2014; Knodel & Shrestha, 2018). They live in the soil, dead wood-above ground or underground, and feed on animals, soil organic matter and plants. The soil dwelling larvae of click beetles cause damage to different parts of the plant (roots, root nodules, stems, leaves, flowers, pods, and seeds) (Knodel & Shrestha, 2018) and in many plants including carrots, sugar beet (Laznik et al., 2014), potatoes and maize. They reduce yields and

affect the value of crops (Ansari et al., 2009; Vernon et al., 2013; Traugott et al., 2014; Barsics et al., 2013; Keiser et al., 2012; Parker and Howard, 2001; Ritter and Richter, 2013; van Herk and Vernon, 2014; Vernon and van Herk, 2013). Some studies reported that it is challenging to control wireworms, mainly because of the long life cycle of wireworms, their distribution in the soil for many years, making it difficult to access them (Studacher et al., 2013; Blackshaw and Kerry, 2008; Saussure et al., 2015; Blackshaw and Hicks, 2013; Blackshaw and Vernon, 2006). To address the wireworm issues and control the pest, growers have been using chemical pesticides such as neonicotinoids, pyrethroids, as well as a phenyl pyrazole to reduce crop damage. However; chemical pesticides can affect human health and the environment. Consequently, their use has been prohibited in several countries (Saussure et al., 2015; Geiger et al., 2010; Traugott et al., 2015). Therefore, the use of environmentally friendly plant protection techniques are in demand as an alternative to chemical control measures (Saussure et al., 2015; Staudacher et al., 2013; Laznik et al., 2014). There are different strategies to control wireworm such as field avoidance (avoid planting immediately after removing grassland), field management practices such as cultivation and crop rotation (Alyokhin et al., 2013). According to a recent study using crop rotation in potato fields in Prince Edward Island, potato rotation with brown mustard or buckwheat was shown the decrease populations of wireworm (Alyokhin et al., 2013; Noronha, 2011). In addition, corn and soybean as rotation crops have been shown to limit rootworm populations. Control of wireworm using crop rotation is sustainable and environmentally friendly compared to chemical pesticides (Noronha, 2011). It appears that some plants have the ability to maintain and protect themselves using some mechanisms that occur naturally with the assistance of soil microbes above or underground. As indicated by Ortiz-Castro et al. (2009), interactions exist between plants and the soil microbiome, and understanding these plant-microbiome interactions can lead to improved agricultural production systems (Ortiz-Castro et al., 2009). It is also known that plants produce a wide range of organic compounds including sugars, organic acids and vitamins, that can be used as nutrients or signals by microbial populations above and belowground (Sausa et al., 2015; Ortiz-Castro et al., 2009). On the other hand, microorganisms in the soil release phytohormones, small molecules or volatile compounds, which may act directly, or indirectly to activate plant immunity or regulate plant growth and morphogenesis (Ortiz-Castro et al., 2009). Thus, this study will focus on examining the role of elucidating soil microbial diversity in the buckwheat's rhizosphere compared with other rotation crops using metagenomics.

Hence, this literature review will first discuss the importance of crop rotation, the relationship between crop's rhizosphere and their ability to host soil microbiomes as well current methods for soil microbial diversity studies.

1.1.1 Crop rotation as an integrated pest management (IPM) tool

Integrated pest management (IPM) is a pest control program that integrates all available tools to protect agricultural crops from insects, weeds and diseases by reducing pest populations to a tolerable level in the most economic and environmentally friendly context (Dee Ann Benard, 2012). These tools include cultural, mechanical, biological and chemical pest control measures, as well as regular pest monitoring. The key observation is that most of these tools do not involve pesticides. IPM programs make extensive use of the information collected in the cropping system and require careful management by the grower. In the IPM process, it is important to understand the biology and behavior of the pest, beneficial organisms as well as monitoring and diagnostic techniques to implement an IPM program. Once the pests have been identified for a crop, the growers should determine the available management techniques such as chemical pesticides or crop rotation. The most important thing for growers willing to use chemical pesticides is to determine what pest-control products are registered and available for the crop of interest. When there are no registered chemicals available and damage cannot be tolerated, the only option will consist of various non-chemical methods for pest control. If used correctly and effectively, these techniques can reduce pest populations in many situations (Larkin and Honeycutt, 2012). Among these techniques, crop rotation is able to keep levels of certain damaging soil insects and diseases at a lower level. Although it may not be regarded as a technologically innovative tool, crop rotation appears to be one of the most effective tools to prevent pest problems and a more reliable and economical management strategy (Thompson, 2014; Larkin and Honeycutt, 2012). There are several pest management strategies for improving soil productivity. Management practices such as tillage and crop rotation, periodic fertilization, and pesticide application generate temporal and spatial changes in the physical and chemical properties of agricultural systems and soils (Al-Kaisi et al., 2003; Trivedi et al., 2016). Moreover, it has been confirmed that crop rotation is an effective strategy for addressing environmental concerns (Al-Kaisi et al., 2003; Larkin and Hon-

eycutt, 2012; Furlan and Kreutzweiser, 2014). In fact, crop rotation has many environmental and economic benefits because it: (1) improves yield and productivity over time; (2) controls weeds, breaks disease cycles; (3) limits insect and other pest infestations; (4) provides an alternative source of nitrogen; (5) reduces soil erosion and increases soil organic matter; (6) reduces runoff of nutrients and chemicals, as well as the potential for contamination of surface water (Al-Kaisi et al., 2003; Larkin and Honeycutt, 2012). Recently, the benefits of crop rotation to manage wireworm populations have been reported (Esser et al., 2015; Furlan and Kreutzweiser, 2014). According to Furlan and Kreutzweiser (2014), wireworm populations are influenced by several factors such as crop rotation, available food resources in the soil, soil characteristics as well as soil organic matter. However, some studies conducted in Italy reported that crop rotation is the most important factor affecting the level of wireworm population densities (Furlan and Kreutzweiser, 2014).

1.1.2 Plant rhizosphere

In 1904, Loranze Hiltner was the first to describe use the word rhizosphere, which was derived from the Greek word "*rhiza*" meaning root, and "sphere" meaning field of influence (Morgan et al., 2005). Plant rhizosphere is the narrow portion of the soil around plant roots that is influenced directly by root secretions and associated soil microorganisms (Nihorimbere et al., 2011; Jacoby et al., 2017). The rhizosphere is therefore home to an overwhelmingly large amount of microorganisms and invertebrates and is considered to be one of the most dynamic interfaces on earth (Philippot et al., 2013). Microorganisms that are present in the rhizosphere interact with plant roots in different ways, as there can be positive, negative or neutral interactions, which can influence plant development and growth (Morgan et al., 2005; Philippot et al., 2013; Jacoby et al., 2017).

The rhizosphere can be divided into three regions: (1) the endorhizosphere which is the root tissue including the endodermis and cortical layers; (2) the rhizoplane which is the surface of the root including the epidermis and mucilaginous polysaccharide layer; and (3) the ectorhizosphere which is the "outer zone" (the soil nearby the root) that extends from the rhizoplane out into the bulk (representing the rhizoplane together with closely adhered rhizosphere soil) (Badri

et al., 2009; McNear Jr., 2013; Sarhan et al., 2018). In addition, plants colonized by mycorrhizal fungi have a region called the mycorrhizos phere (McNear Jr., 2013; Ahkami et al., 2017; Morgan et al., 2005).

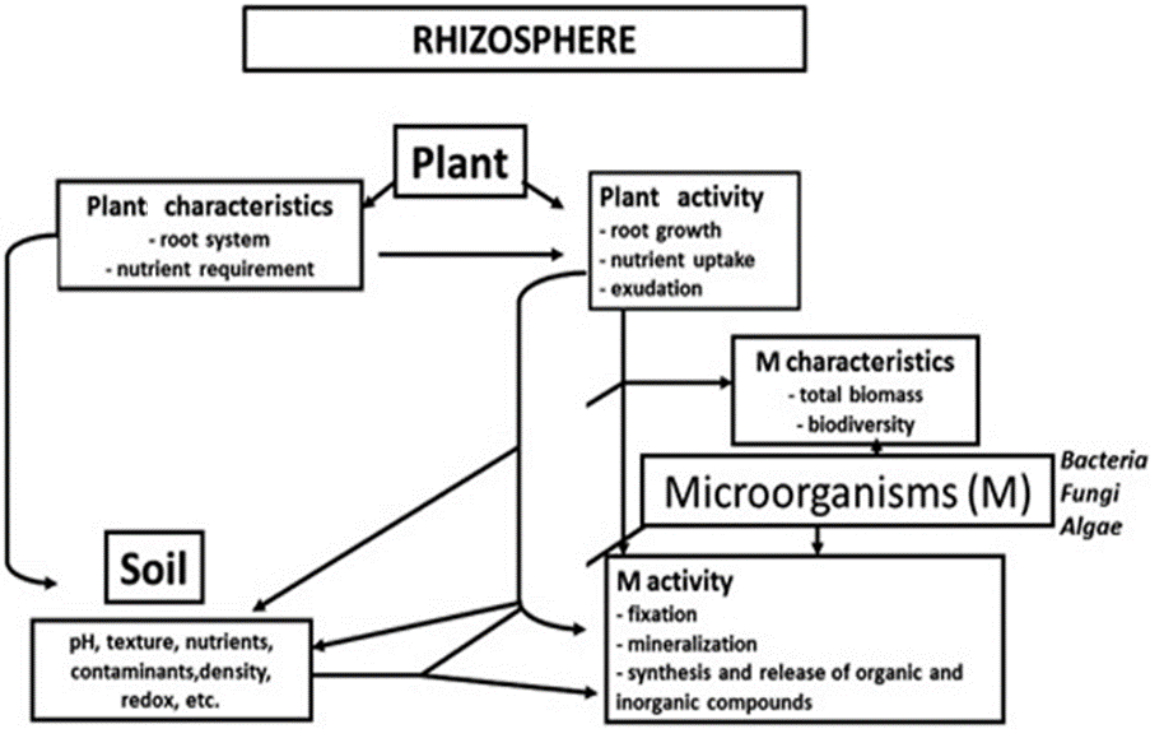

Figure 1. Description of plant rhizosphere and its interactions (Gianfreda, 2015)

1.1.3 Role of plant rhizosphere microbiome in plant health

Rhizosphere microbiotas play an essential role in the functioning of plant growth and health. These microbiotas include bacteria, fungi, oomycetes, viruses, nematodes, and archaea, and these microorganisms are a part of a complex trophic web that utilizes a massive amount of nutrients released by the plant roots. In fact, the rhizodeposits such as exudates, border cells and mucilage are the main driving forces in the regulation of microbial diversity and activity taking place on plant roots (Philippot et al., 2013; Souza et al., 2015). Rhizosphere microorganisms are capable of both directly and indirectly influencing the composition, diversity, and productivity

(biomass) of plant communities (Morgan et al., 2005). Thus, the belowground microbial species have been suggested as an indicator of aboveground plant diversity and productivity; these species may play key roles in maintaining plant productivity under various environmental conditions. Many studies have shown the beneficial impact of rhizosphere microbes on plant growth, health and disease resistance, suggesting that microorganisms in the rhizosphere promote plant growth and protect plants from pathogen attacks by different mechanisms (Morgan et al., 2005; Jacoby et al., 2017). These mechanisms involve bio fertilization (which is a live formulation of micro-organisms used to fertilize farm lands as a nitrogen fixation source and includes bacteria and fungi), stimulation of root growth, rhizoremediation which refers to the clean up of the harmful pollutants found in the soil by rhizomicroflora (Oberai and Khanna, 2018), control of biotic stress, as well as disease control (Mendes et al., 2013). Some of these mechanisms have been well described and established for Rhizobacteria belonging to the *Proteobacteria* and *Firmicutes* (*Pseudomonas* and *Bacillus*) as well as for fungi from the *Deuteromycetes* (*Trichoderma* and *Gliocladium*) (Mendes et al., 2013). Widely known examples include nitrogen-fixing bacteria, mycorrhizal fungi that facilitate phosphorus uptake (Mendes et al., 2013), and *Pseudomonas fluorescens* that produces the antifungal compound diacetylphloroglucinol (DAPG) (Tuner et al., 2013), playing essential roles in the suppression of a wide range of plant diseases. Some studies demonstrated that the increase of antagonistic activity of *Pseudomonas* spp. that controls specialized pathogens increased disease suppression in soils (Linkun et al., 2015; Chen et al., 2017).

1.1.4 Microflora of the rhizosphere

The rhizosphere microflora consists of fungi, bacteria, nematodes, protozoa, algae and micro arthropods (Nihorimbere et al., 2011). About 98% of the soil microbes cannot be cultured and are thus difficult to identify, characterize, and describe (Nihorimbere et al., 2011). However, nucleic acid-based techniques such as the analysis of DNA and rRNA molecules from soil samples have allowed the discovery of a massive variety of microbial flora in the rhizosphere (Nihorimbere et al., 2011; Suzuki et al., 2006), and provided an opportunity to study a much broader range of microorganisms residing in the rhizosphere (Gabriele and Kornelia, 2009). According to Nihorimbere and collaborators (2011), there is a high number of microbial species present in the

soil, and the most abundance species found so far in the rhizosphere are *Proteobacteria* and the *Actinobacteria* (Mendes et al., 2013*)*. Other studies indicated that the composition of bacterial community in the rhizosphere is affected by a complex interaction between soil type, plant species and root zone location (Gabriele and Kornelia, 2009; Morgan et al., 2005). Moreover, the major factors that determine the composition of the microbial communities in the rhizosphere have been reported to be the plant species, plant developmental stages, and soil type (Liang et al., 2014; Linkun et al., 2015; Wang et al., 2017) and the relationship between plant species and their productivity has further been suggested to be influenced by their interactions with microorganisms existing in their rhizospheres (Philippot et al., 2013; Linkun et al., 2015).

1.1.5 Main characteristics of the root exudation process

The release of organic compounds from living plant roots into the surrounding soil is known as root exudation or rhizodeposition and it occurs through at least two potential mechanisms. The first is the leakage of compounds over which the plant exerts little control and the second is the exudation of specific compounds under the control of the plant (Hunter et al., 2014). The exudation rates *sensu stricto* vary extensively among species and environmental conditions (Lambers et al., 2009). The exudates are transported across the cellular membranes of root cells and secreted into the surrounding rhizosphere. Plant natural products are also released from the border cells of roots (Bais et al., 2006). Nevertheless, it is very difficult to identify root exudates with respect to their chemical composition and concentration in the soil because of methodological limitations (van Dam and Bouwmeester, 2016; Nihorimbere et al., 2010). In fact, the released organic materials are subject to microbial attack and degradation at the moment of exudation and, thus cannot be subsequently enriched and separated from the roots in the natural environments. Thus, root exudation has been quantified by measuring the production of labelled CO_2 in the rhizosphere from ^{14}C-labelled plants, and 12-40% of the total carbohydrates produced by photosynthesis is estimated to be released into the soil surrounding the roots (Nihorimbere et al., 2011; Brimecombe et al., 2007). Typically, according to Nihorimbere et al. (2011) root exudates can consist of water, soluble sugars, organic acids, amino acids, as well as hormones, vitamins, amino compounds, phenolic, and sugar phosphate esters (Nihorimbere et al., 2011). How-

ever, the composition of root exudates has been reported to be affected qualitatively and quantitatively by different environmental factors such as pH, soil type, oxygen status, light intensity, soil temperature, nutrient availability and the presence of microorganisms, which might have a great influence on root exudation rather than variances due to the plant species (Singh et al., 2004; Nihorimbere et al., 2011). Several plant species have been found to increase the rates of carboxylate exudation once their phosphorus (P) supply are severely limited and higher exudation rates have been reported for species producing root clusters at low P levels (Lambers et al., 2009; Linkun et al., 2015). Root exudation is also dependent on the growth stages and the nutrition status of the plant (Carvalhais et al., 2013; Costa et al., 2006; Bais et al., 2006). Some nutrients such as K+, Na+ and Mg++ are known as essential components of major enzymes that regulate major biological processes in plants (Carvalhais et al., 2013). Therefore, low nutrient availability can be a major constraint for plant growth in many environments, especially for the soils extremely deficient in these micronutrients such as B,Cu, Fe, Cl, Mn, Mo, and Zn. As a consequence, some species have been found to typically exudate organic acids or phytosiderophores in response to P and Fe or Fe and Zn deficiencies (Nihorimbere et al., 2010). According to Bais et al. (2006), root exudates play an important role for decreasing the pathogenic infection.

1.2 Mechanisms of plant/rhizosphere microbiome interactions in plant health

1.2.1 Beneficial microorganisms and modes of action

The interactions between plant and beneficial microbial organisms can be divided into three categories: 1) the microorganisms that are associated with the plants and involved in its nutrition, such as, the microorganisms able to increase the supply of mineral nutrients to the plants. However, the majority of them might not interact directly with the plants but still affect the growth of plants; 2) the microorganisms that promote plant growth indirectly by hindering the growth or activity of pathogens; and 3) the microorganisms directly responsible for promoting the growth of plants (Umesha et al., 2017; Ortiz-Castro et al., 2009).

1.2.2 Plant growth promoting rhizobacteria (PGPR)

Plant growth promoting bacteria are known as soil bacteria that enhance plant growth and are usually associated with the above and underground plant parts (roots, leaves, and flowers) but also within the plant tissues (Trujillo et al., 2015; Glick, 2015). Plant growth promoting bacteria have positive effects on plants either directly by facilitating the acquisition of resources such as N (through fixation), P, Fe, controlling plant hormone levels, or indirectly by protecting plants from diseases such as biocontrol agents (Trujillo et al., 2015). The rhizosphere supports various bacteria that stimulate the growth of plants such as plant growth promoting rhizobacteria operating through a wide variety of mechanisms (Morgan et al., 2005). Root-associated beneficial soil bacteria are well known to promote plant growth and enhance plant production (Vurukonda et al., 2018; Bargaz et al., 2018). PGPR were first defined by Kloepper and Schroth (1978) as organisms that, after being inoculated on seeds, can successfully colonize plant roots and positively enhance plant growth (van Loon et al., 1998; 2007). PGPR grow on, in, or around root plant tissue and improve plant growth, increase yield, defend plants against pathogens, and decrease abiotic or biotic stress. Growth promotion can be achieved directly via the interaction between the microbe and the host or indirectly through antagonistic activities against plant pathogens (Bargaz et al., 2018). According to Vurukonda et al. (2018), rhizobacteria and mycorrhizal fungi are among the beneficial microorganisms that enhance plant growth and production. A number of plant-interacting microbes produce phytohormones to prevent or promote root growth, protect plants against biotic or abiotic stress, and enhance nutrient acquisition by the roots. PGPR represent an environmentally friendly alternative for increasing crop production and plant health (Vurukonda et al., 2018). Thus, PGPR are expected to reduce the need for chemical fertilizers and chemical pesticides (Rincon-Florez et al., 2013; Vurukonda et al., 2018) because of the interactions that occur between some rhizobacteria and plant roots (Bargaz et al., 2018). For example, *Pseudomonads* have been characterized as plant growth promoting rhizobacteria (Morgan et al., 2005), and to date, over two dozen genera of non-pathogenic rhizobacteria have been identified. Plant growth promotion can also occur indirectly through the control of pathogens (biocontrol) by the PGPR via mediated induced systemic resistance (ISR) through the synthesis of antibiotics consisting mainly of secondary metabolites (van Loon et al., 2007; Vurukonda et al., 2018). The density and structure of the microbes on the root surface have been reported to be de-

pendent on nutrient availability and physicochemical variations on the surface of the root (McNear Jr., 2013). The same author mentioned that root exudates can serve as a food source and chemoattractant for microbes attached to the root surface and forming micro-colonies. Thus, epidermal cell junctions, root hairs, axial groves, cap cells, and sites of emerging lateral roots have been reported as the common sites for bacterial attachment and colonization. Micro-colonies grow later into larger biofilms forming multiple layers of bacteria encased into an exo-polymeric matrix (McNear Jr., 2013).

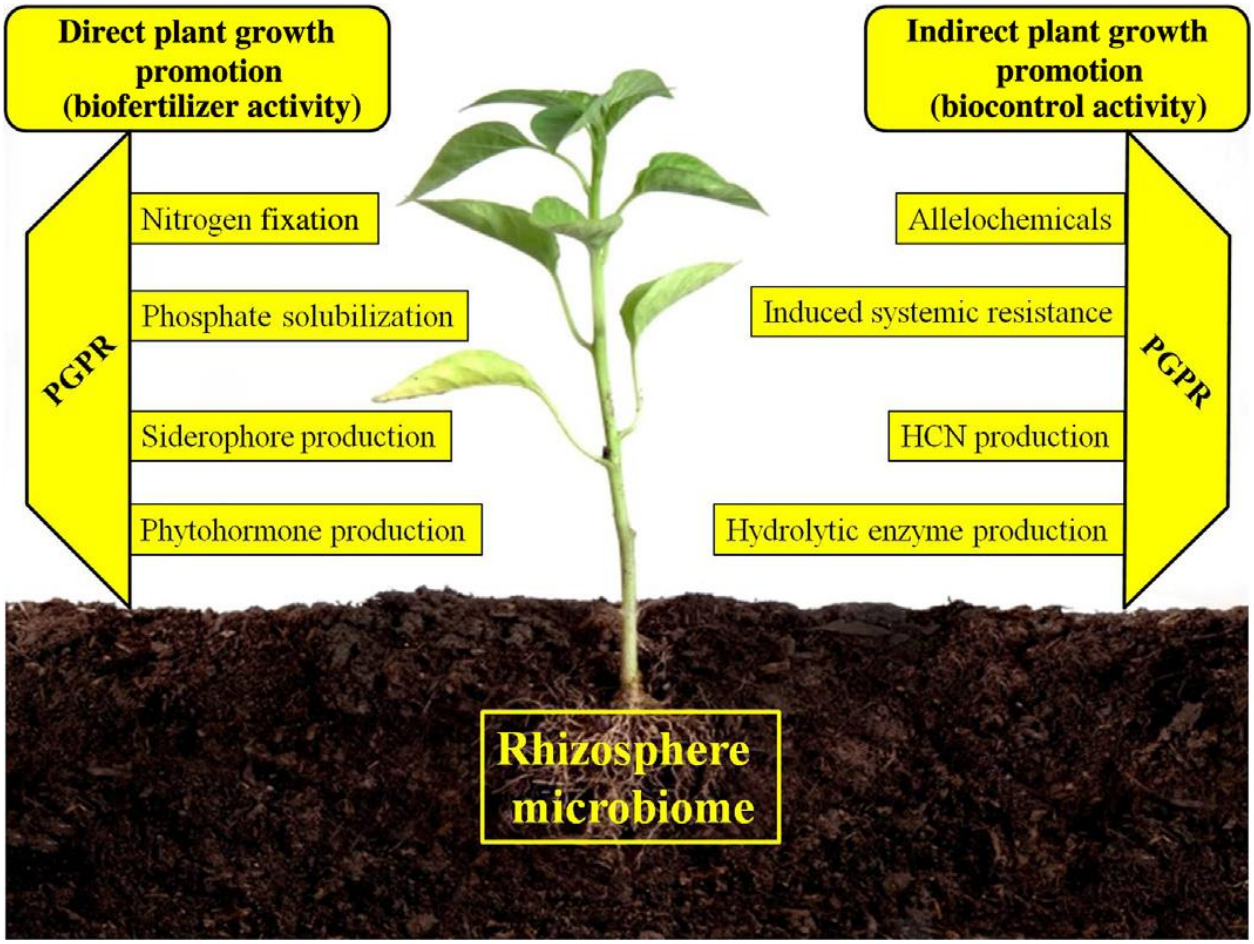

Figure 2. Mechanism of action of Plant Growth Promoting Rhizobacteria (Umesha et al., 2017)

1.2.3 Plant species and microbiome community structure

According to Rincon-Florez (2013), plants have the ability to shape their rhizosphere microbiome structure. Some plant species have been shown to host specific communities and attract defensive microorganisms to suppress pathogens in the rhizosphere (Linkun et al., 2015). Morgan et al. (2005) reported that the species and genotype of the plant also determine the types of members and assemblages of microorganisms that can grow and multiply in the rhizosphere, and can strongly influence the composition and activity of such microorganisms. These species-specific effects have been attributed to differences in root morphology, and amount and type of rhizodeposits among plant species and genotypes (Philippot et al., 2013). It is also known that specific metabolites released into the rhizosphere can trigger multiple responses in different soil microorganisms. For example, plant flavonoids can attract symbionts such as *Bradyrhizobium japonicum*, and pathogens such as *Phytophthora sojae* (Philippot et al., 2013). Flavonoids have also been reported to stimulate mycorrhizal spore germination and hyphal branching as well as influencing quorum sensing (Philippot et al., 2013; Mendes et al., 2013; Faure et al., 2009; Guo et al., 2011; Hassan and Mathesius, 2012; Pérez-Montaño et al., 2011). Likewise, constitutive secondary defence metabolites including pyrrolizidine alkaloids have been reported to affect the rhizosphere microbiota by favouring resistant or tolerant microorganisms, or in some cases, microorganisms that metabolize these compounds (Philippot et al., 2013; Callaway et al., 2008; Mendes et al., 2013).

1.2.4 Rhizosphere plant-microbial interactions

The rhizosphere is one of the most complex environments involving many interactions playing crucial roles in plant health. It is known that plants secrete up to 40% of their photosynthates reaching the roots and their rhizosphere microbiomes (Rincon-Florez et al., 2013).

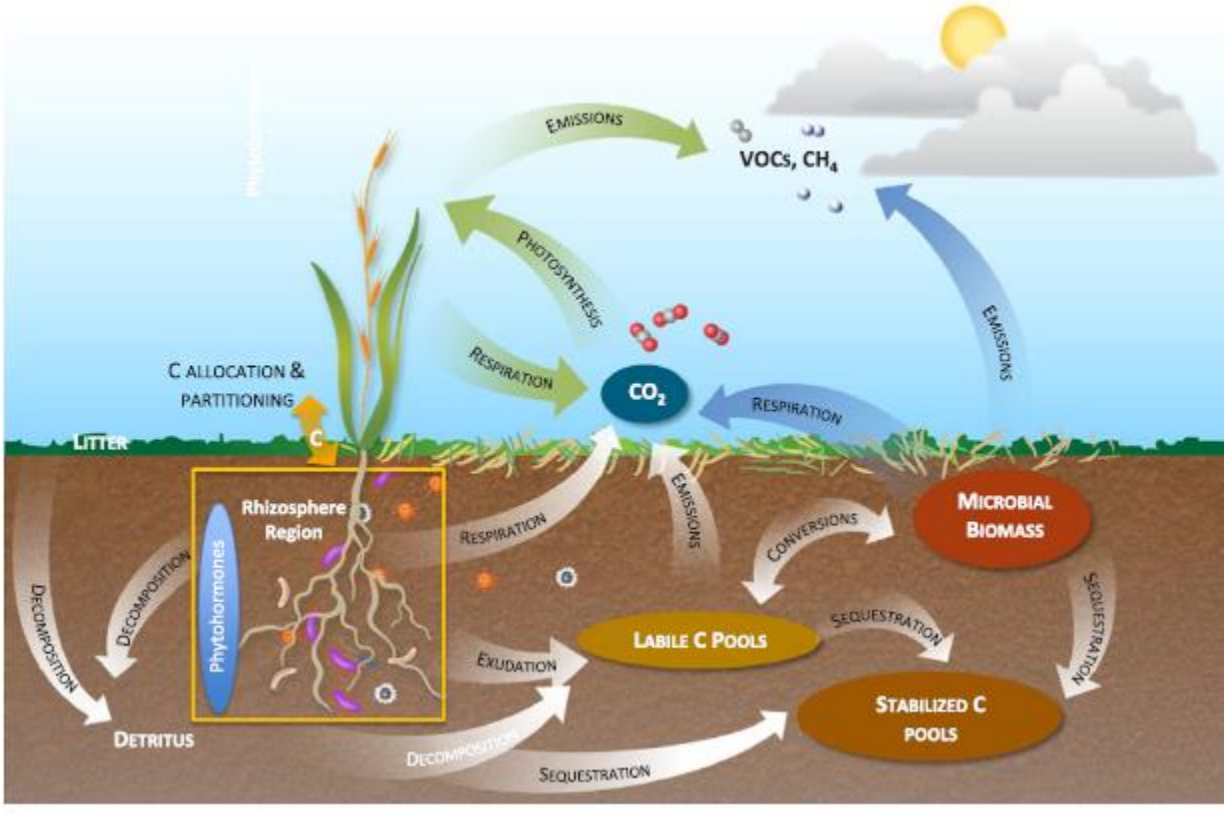

Figure 3. Demonstration of interactions between the rhizosphere and the surrounding ecosystem (aboveground and belowground) and their effects on carbon flow. Different pathways of entry and exit for carbon are shown. Root exudation, root breakdown or aboveground biomass constitutes major sources for entry of carbon. Carbon exits also in the soil as direct carbon emission sources in the form of root biomass microbial biomass, volatile organic compounds (VOCs) or methane, some which constitute carbon exit sources (Ahkami et al., 2017).

1.2.4.1 Multi-trophic interactions in the rhizosphere

The rhizosphere, which is defined as the narrow zone of soil surrounding the roots of plants has a wide variety of microbial organisms that affect plant growth, nutrition as well as plant health (Philippot et al., 2013). The direct and indirect interactions between hosts and their associated microbiota involve constitutive and inducible alterations of secondary metabolism.

Secondary metabolites are organic compounds released by plants, fungi and microbes, that are derived from primary metabolites, but not required for plant growth and development of the producing organism. Plant primary metabolites are the primary products such as nucleic acids, proteins, carbohydrates, fats and lipids, which are a critical for development and homeostasis (Philippot et al., 2013). Communication through signalling molecules, such as flavonoids, strigolactones and sesquiterpenes is important for the regulation of these interactions. In some interactions between plant and microbiome, flavonoids have been reported to play a key role as signaling components (Shaw et al., 2006; Badri et al., 2009). In addition, when plant roots release strigolactones, the metabolites stimulate the growth of arbuscular mycorrhizal fungi (AMF) as well as parasitic plants such as *Orobanche* spp. (Philippot et al., 2013; Rasmann et al., 2005). As mentioned earlier, interactions in the rhizosphere have been reported to affect plant health. Pathogenic bacteria, fungi including AMF, oomycetes, nematodes and micro arthropods have adverse effects on plants (Philippot et al., 2013). According to Badri et al. (2009), fungi and nematodes are major players in the soil in terms of prevention of disease (Badri et al., 2009). Viruses can also infect plants via the roots but require nematodes or fungi to penetrate the root tissue (Philippot et al., 2013). Rhizodeposits are essential signals for germination, chemotaxis and directional growth of pathogens towards the plant roots. For instance, the bacterial pathogen *Agrobacterium tumefaciens* is attracted by particular phenolic compounds (acetosyringones) released from wounded plant tissue (Philippot et al., 2013). Additional phenolic compounds such as vanillic acids in the root exudates are able to trigger spore germination of fungal pathogens. For the oomycetes, it was demonstrated that electro-taxis is an important root-targeting mechanism for motile zoospores. Oomycetes and other pathogens can also capture symbiotic signalling molecules, such as cutin monomers to trigger the initiation of infections (Philippot et al., 2013).

1.2.4.2 Belowground – aboveground interactions

There have been many studies conducted to illustrate how below-ground interactions can affect above-ground communities of carnivores, herbivores, mutualists as well as symbionts (Philippot et al.. 2013). These belowground – aboveground interactions may occur due to altered nutrient or water uptake by the plant, or altered plant defences (Philippot et al., 2013; Gamborena

et al., 2005; Staley et al., 2007). Fungi living in the rhizosphere, including the mycorrhizal fungi (*Glomus intraradices*) (Veiga et al., 2011), and rhizobacteria, such as *Bacillus* spp. and *Pseudomonas* spp. can stimulate the resistance response of the plant, improve health and yield of different crops, and are typically efficient against multiple pathogens and insect pests (Zamioudis and Pieterse, 2012; Bargaz et al., 2018). Conversely, resistance responses that are induced in the phyllosphere can be distributed consistently to the roots, affect the microbial diversity of the rhizosphere, and influence the interactions in the canopy aboveground (Bais et al., 2006). The systemic resistance of plants has been found to be influenced by various non-pathogenic rhizobacteria that prime the plant for activation of various cellular resistance responses induced during pathogen attacks. The systemic resistance responses are dependent on the inducing microorganism, are regulated by the plant hormones jasmonic acid, salicylic acid and ethylene, and can lead to an oxidative burst leading to hypersensitive response (HR), the production of secondary metabolites and/or cell wall reinforcement (Philippot et al., 2013).

1.3 Soil microbial diversity

Soil microbial communities or microbiomes are crucial for the health of the environment. These microbial communities drive major geochemical cycles and aid to maintain plant health (Trivedi et al., 2016; Tom et al., 2012). As reported by Tom et al. (2012), interactions and metabolic exchanges occur among members of the microbial community and their ecosystem. However, there is still a lack of understanding of the mechanisms of action. In this context, the studies performed on microorganisms by either culturing soil microorganisms or sequencing the 16S rRNA gene demonstrated the phylogenetic and functional diversity, metabolic potential and evolutionary adaptations within communities (Tom et al., 2012). According to Jacqueline et al. (2012), there are different plant factors that influence microbial communities. These include plant age, plant species (Linkun et al., 2015), plant genotype, and root exudates (Castro-Sowinski et al., 2007; Liang et al., 2014). Soil structure and soil microbial diversity can be influenced by soil organic matter (Carter et al., 2009). It has been reported that one gram of soil can contain more than 10,000 bacterial or fungal species. These microbial communities have been connected with soils of varying texture, N and P content as well as soil pH. However, soil pH has been

shown to have the most impact on the soil microbial community because of the sensitivity of bacterial cells to pH. In addition, root exudates have been reported to influence the composition of soil microbial communities (Jacqueline et al., 2012; Castro-Sowinski et al., 2007; Liang et al., 2014).

1.3.1 Methods for studying soil rhizosphere microbiome

Microbes are essential to maintain life on Earth (Turner et al., 2013). Therefore, studying and understanding the microbial diversity in different environments including soils, oceans, human and animals is important to further our understanding of biological and evolutionary processes. In the last decades, the study of soil microbial communities relied on laboratory culturing techniques, phospholipid fatty acid analysis (PFLA), denaturing gel gradient electrophoresis (DGGE), and terminal restriction fragment length polymorphism (Coats and Rumpho, 2014). Culture-based approaches revealed that only about 1% of visible microscopic cells can be cultured using conventional techniques. Recently, next generation sequencing technologies have made it possible to generate megabase of sequence data in a matter of hours which has enabled scientists to model the structure and composition of microbial communities (Coats and Rumpho, 2014). The major limitation of next generation sequencing technologies lies in the handling and managing of the expansive datasets and the application of appropriate statistical analyses to address the biological questions at hand (Coats and Rumpho, 2014). While culture-based techniques have allowed the isolation of microbes for detailed studies, molecular techniques such as metagenomics are increasingly enabling the identification of microbes. The microbial communities or microbiomes from various environments have been studied in this way in order to understand their ecological function (Turner et al., 2013).

1.3.2 Soil sampling methods

Soil sampling provides a clear image of what organisms are present in the field. If the sampling is not completed in an accurate way, it will affect the accuracy of the outcome. In addition, the optimal number of the replicates should be considered because it will enhance the accuracy

of the generated data (Carter and Gregorich, 2007). Soil sampling is a fundamental farm management tool for determining soil fertility and understanding some of the phenomena occurring in the soil. A good soil fertility program requires regular soil sampling, plant tissue testing, precise record keeping based on accurate farm map coordinates of each sample, and best crop management practices associated with each field (Rovira-Más and Sáiz-Rubio, 2013). The sample pattern type should be considered to obtain an accurate soil test (Zorzia et al., 2008). Depending on the purpose of the study, different soil sampling and collection methodologies have been proposed, and include judgement sampling, simple random sampling, stratified random sampling, systematic sampling. Among these methods, random sampling and systematic sampling are widely used in environmental studies (Carter and Gregorich, 2007). Consequently, a random and systematic sampling strategy was adopted in the current study.

1.3.3 Molecular methods

The latest improvement in next generation sequencing (NGS) technologies has enabled researchers to better understand microbial diversity through DNA genome sequencing. Using these NGS platforms, several applications ranging from single-gene targeted sequencing to whole-genome sequencing and shotgun metagenome sequencing have been reported to study the microbial population in a culture-independent context (Kim et al., 2013). These techniques employ sequencing methods such as 454 pyrosequencing sequencing, whole genome shotgun sequencing, or 16S rRNA amplicon sequencing using technologies including Illumina or Ion Torrent sequencing platforms (Petrosino et al., 2009).

1.3.3.1 16S rRNA amplicon sequencing

The ribosomal 16S RNA (16S rRNA) gene has been widely used for species identification, classification of uncultured bacteria, and taxonomic analysis of microbial diversity (Petrosino et al., 2009; Vetrovsky and Baldrian, 2013). Moreover, 16S rRNA sequencing is becoming the most common procedure for the identification of the environmental microbial diversity due to the presence of several hypervariable regions (V1-V9) within the bacteria 16S rRNA (Figure 4) (Vetrovsky and Baldrian, 2013; Rosselli et al., 2016). It was first used for bacterial evolutionary and phylogenetic studies in the 1980s (Petrosino et al., 2009; Cheng et al., 2014; Vetrovsky and

Baldrian, 2013).

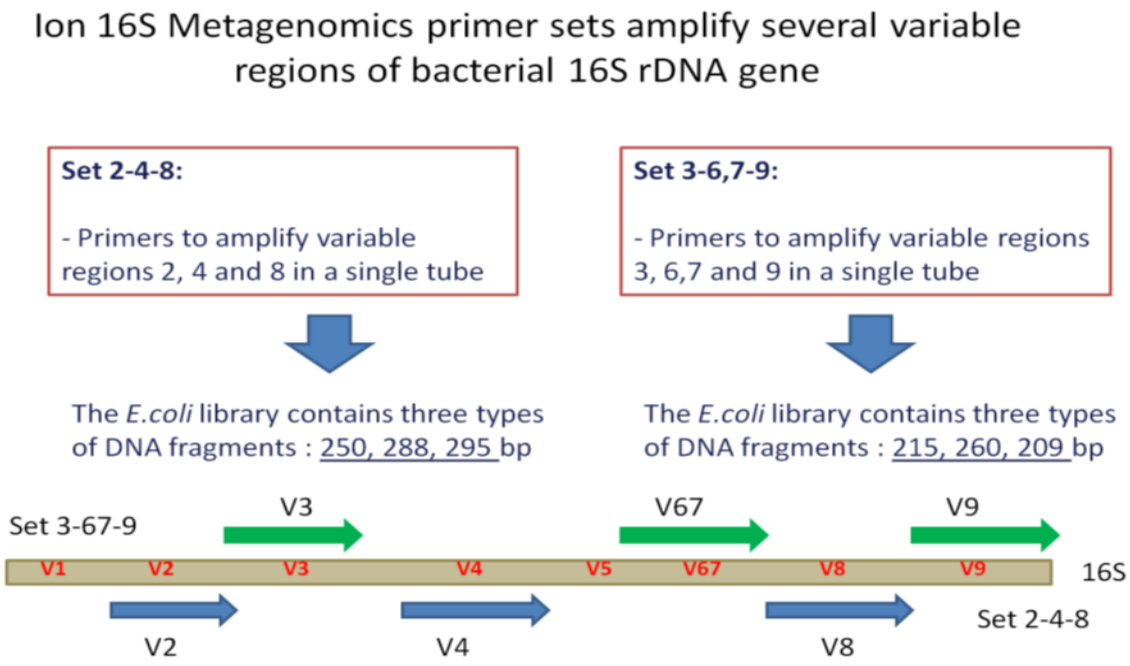

Figure 4. Schematic representation of the 16S Metagenomics Primer Pool sets, targeting the hypervariable regions of 16S DNA. Two sets of primers pools can simultaneously be used to amplify 7 of the 9 hypervariable regions of the 16S rDNA gene in bacteria: Pool 1: primer set for the amplification of V2-4-8 hypervariable regions; Pool 2: primer set for the amplification of the V3-6,7-9 hypervariable regions.

1.3.3.2 Whole genome metagenomic shotgun sequencing

Whole genome metagenomic sequencing techniques, considered as an extension of 16S rRNA amplicon sequencing techniques, improved the throughput of sequencing systems and identification of microbial diversity in variant populations (Petrosino et al., 2009). Whole genome metagenomic approaches empower researchers to distinguish and interpret various collections of microbial genes encoding varied biochemical or metabolic functions (Petrosino et al., 2009). According to Yooseph et al. (2007), the results of studies conducted on marine samples

using whole genome shotgun sequencing enabled the discovery of new genes and functions such as the discovery of novel protein families (Petrosino et al,. 2009; Yooseph et al., 2007).

1.3.3.3 454 Pyrosequencing Sequencing

According to Petrosino et al. (2009), pyrosequencing has been utilized in many different studies such as genotyping, detecting single nucleotide polymorphism (SNP), as well as assessing microbial diversity (Marsh, 2007; Petrosino et al., 2009). Over the last decade, 454 pyrosequencing has been the most common method of generating amplicon metagenomics data among NGS platforms due to its capability of generating longer read lengths compared to short read technologies like illumina and Ion Torrent (Petrosino et al., 2009; Kim et al., 2013). DNA pyrosequencing (sequencing-by-synthesis) was developed in the mid-1990s as a different method to Sanger DNA sequencing (Petrosino et al., 2009). The major limitation of pyrosequencing resides in its inability to generate reads longer than 1000 bp (Mayo et al., 2014). Pyrosequencing has been effectively applied to microbial diversity studies due to its ability to facilitate microbial identification by sequencing multiple hypervariable regions within 16S rRNA gene and gene signature sequences (Petrosino et al., 2009; Sharma et al., 2008) and with high accuracy (Kim et al., 2013). However, the high cost per mega base (MB) read as well as difficulties in sequencing homopolymer stretches are the main weaknesses of the 454 platform (Mayo et al., 2014).

1.3.3.4 Illumina and Ion Torrent PGM sequencing platforms

Next generation sequencing (NGS) technologies and associated bioinformatics approaches are the latest advances that enable scientists to understand the bacterial diversity in any environment (Rincon-Florez et al., 2013). Currently, there are two major NGS platforms widely used in metagenomics: Illumina (Illumina, San Diego, CA, USA) and Ion Torrent NGS systems (ThermoFisher Scientific, Waltham, MA, USA). Although each NGS platform is unique in how sequences are generated and data analyzed, the Ion Torrent PGM and the Illumina MiSeq are closely related and share similar methods for library and template preparation, and data analysis. The Ion Torrent PGM sequencing system is light independent where sequence composition is determined by measuring pH changes due to hydrogen ion liberation as nucleotides are incorporated during strand synthesis in picolitre wells (Grada and Weinbrecht, 2013; Rincon-Florez et

al., 2013). Conversely, the Illumina MiSeq sequencing system relies on the detection of fluorescence generated by the incorporation of fluorescently labeled nucleotides into the growing strand of DNA (Grada and Weinbrecht, 2013). Ion PGM sequencing has been widley used to assess bacterial and archaeal community dynamics and structure. For example, it has been used to discribe the microbial community involved in diesel biodegradation (Rincon-Florez et al., 2013).

Illumina Miseq sequencing has been reported to produce many more reads, at a cheaper price and with more accuracy (accuracy of >99%) than 454 pyrosequencing platforms (accuracy of 98.93%), but is somewhat limited in certain fields due to the relatively short read lengths (<100 bp) generated by early versions of this technology (Kim et al., 2013). Mayo et al. (2014) reported that the Illumina platform generates 150 – 300 bp pair-end reads whereas the Ion Torrent platform is able to generate up to 400 bp single read within 7 h at moderate cost, making Ion Torrent technique more useful in some applications (Mayo et al., 2014).

1.4 16S RNA metagenomics by next generation sequencing as a proposed approach

The majority of microorganisms live in communities (aggregations of multiple species), many of which are relatively complex, containing thousands of interacting members (Niu et al., 2017). About 98 % of these community members cannot be cultured, making the study of microbial diversity challenging and complicated (Nihorimbere et al., 2011). However, metagenomics or the culture-independent genomic analysis of an assemblage of microorganisms have allowed scientists to answer several hypotheses and questions with regard to microbial ecology, thus providing novel tools for studying uncultivable microorganisms (Boon et al., 2013; Handelsman, 2004). Since the 1990s, metagenomics has become one of the fastest growing research areas in microbial ecology, allowing scientists to study the ecological role and the metabolism of microbes in a given ecosystem using environmental DNA directly, in a culture-independent manner (Petrosino et al., 2009). Thus, metagenomics has made significant contributions to microbial ecology as evidenced by the first description of proteorhodopsin among the marine bacteria (Kim et al., 2013; Handelsman, 2004; Knief et al., 2012; Kakirde et al., 2010).

1.4.1 Advantages of the 16S rRNA sequencing strategy

One of the main advantages of functional metagenomics is its ability to identify gene products from uncultured microbes, many of which have no significant homolog within the GenBank database (Kakirde et al., 2011). As mentioned previously, several studies have used metagenomics in diverse environments such as soil, water, and even within animals, including humans (Kakirde et al., 2011). By targeting the 16S rRNA as a marker gene, metagenomics has a wide range of uses: characterization of bacterial populations, taxonomical analysis, and microbial identification (Boon et al., 2013; Hwang et al., 2017). Because the 16S rRNA gene is highly conserved across bacteria and archaea, taxonomic assignment is possible if a sufficient proportion of the nine hypervariable regions (V1-V9) are sequenced and included in the sequencing platform, as is the case for the Ion Torrent PGM platform which uses the nine hypervariable regions (Barb et al., 2016). Other advantages of 16S RNA sequencing includes:

- As 16S rRNA gene is the preeminent taxonomic marker for bacteria and archaea a large database of sequences exists for comparative studies and to facilitate identification of bacterial species when sufficient sequence lengths are achieved (Hwang et al., 2017; Barb et al., 2016; Kim et al., 2013; Garcia-Salamanca et al., 2012).
- Analysis of 9 regions is made possible by the analysis of multiple gene regions. This can be achieved using any sequencing methodology.
- Ribosomes and ribosomal RNA are present in all cells and their sequences are highly conserved in nature (Rusch et al., 2007).
- 16S rRNA sequencing does not require the culturing of microbial cells (Hwang et al., 2017; Barb et al., 2016).

It is evident that using high throughput DNA sequencing technologies makes it possible to study microbial communities without a requirement for cultivation. However, a fundamental challenge in metagenomics has been the evaluation of abundance of organisms in a sample based on the frequency at which an organism's DNA was observed in the reads generated by DNA sequencing (Morgan et al., 2010). To address this, Morgan and his collaborators have developed the following approach: 1) create mixtures of ten microbial species for which the genome sequences are known and include an equal amount of cells for each species in the mixture; 2) ex-

tract and sequence the DNA from the mixtures; 3) determine the frequency of genomic regions from each organism observed in the sequenced DNA (Morgan et al., 2010).

1.4.2 Potential limitations of the strategy

Next generation sequencing is still quite expensive for many laboratories. However, it is cheaper and less time-consuming compared to first generation sequencing (Grada and Weinbrecht, 2013). The startup cost of any NGS platform is more than $100,000, and each sequencing reaction can cost upward of $1,000. Moreover, inaccurate sequencing of homo polymer regions (spans of repeating nucleotides) on certain NGS platforms, including the Ion Torrent PGM, and short-sequencing read lengths (on average 200–500 nucleotides) can lead to sequence errors. In addition, depending on the complexity of the study design, data analysis can be challenging and time consuming. Thus, special knowledge of bioinformatics may be required to gather precise information from sequence data (Grada and Weinbrecht, 2013; Rincon-Florez et al., 2013).

In the current study, a metagenomics approach was undertaken to assess the microbial diversity in the rhizosphere of buckwheat compared with other rotation crops.

1.5 Hypothesis and objectives of the study

1.5.1 Hypothesis

The rhizosphere of different rotational crops hosts different microbiomes. The root system of buckwheat influences the structure of the microbiome in the rhizosphere, and may affect wireworm density in the field.

1.5.2 Objectives

The aim of this study is (1) to determine the microbial diversity in the buckwheat rhizosphere compared with other rotation crops; (2) determine if there is a correlation between the structure of the microbiome of the rhizosphere and wireworm density. This study should provide an indication of how the rhizosphere of buckwheat can impact the soil microbiome and be beneficial to soil health, the cropping system and their impact on wireworms.

CHAPTER TWO: Microbial diversity in the buckwheat rhizosphere in comparison with other rotation crops

2.1 Introduction

The positive impact of crop rotation on soil health and microbial diversity has been reported (Castro-Sowinski et al., 2007). Microbial diversity in cropping systems is mostly influenced by location, rhizosphere effects, sampling times as well as amendment treatments. Some of these effects were found to be associated with soil physicochemical properties such as pH, moisture, organic matter, and nutrient levels (Fernandez et al., 2016). Recent studies showed that microbial diversity plays an important role in soil health and soil fertility (Li et al., 2017). Rhizosphere microbial diversity is affected by different factors including soil type, soil properties, nutrition, management practices (Fernandez et al., 2016; Wu et al., 2008; Mendes et al., 2014), plant age, plant species (Mendes et al., 2014; Linkun et al., 2015), plant genotype (Castro-Sowinski et al., 2007, Garcia-Salamanca et al., 2012), and soil conditions such as organic matter and nitrate concentration. However, Nannipieri et al. (2002) and Yang et al. (2016) reported that microbial diversity is more highly influenced by plants. Different studies report that microbial diversity is affected by plants possibly due to the selective exudation of specific carbohydrates, carboxylic acids and amino acids (Steinberga et al., 2012, Garcia-Salamanca et al., 2012).

The study conducted by Yang et al. (2016) showed that the microbial diversity of rhizosphere soil varies based on crop species and cropping systems (Mahoney et al., 2017). For example, "intercropping peanut with buckwheat, sorghum or glutinous millet was beneficial to the composition of bacterial communities in rhizosphere soils" (Yang et al., 2016). In their study, the authors collected samples from different plants as follow: hulled oat (*Avena sativa*), mung bean (*Vigna radiate*), foxtail millet (*Setaria italica*), barley (*Hordeum vulgare*), rape (*Brassica campestris*), sunflower (*Helianthus annuus*), carrot (*Daucus carota var. Sativus*), flax (*Linum usitatissimum*), naked oat (*Avena nuda*) and tobacco (*Nicotiana tabacum*) as sole-cropped samples. They also collected samples from paired intercropping systems including buckwheat (*Fagopyrum esculentum*)/peanut (*Arachis hypogaea*), glutinous millet (*Panicum miliaceum*)/peanut, peanut/sorghum (*Sorghum bicolor*) and peanut/foxtail millet, to determine any changes in the microbial diversity of rhizosphere soils. Their study demonstrated that the microbial diversity was different in intercropping system compared to sole-crop systems. Therefore, intercropping buckwheat, glutinous millet, and sorghum with peanut might increase the microbial diversity when compared with mono-cropping systems, and several specific bacteria were found to be more

abundant in intercropping systems such as peanut/buckwheat and peanut/glutinous millet (Yang et al., 2016).

Crop rotation and plant diversity are considered as essential factors to maintain soil microbial diversity and activity (Steinberga et al., 2012; Nicolardot et al., 2007; Pascault et al., 2010; Mahoney et al., 2017). In the context of monoculture worldwide (Li et al., 2017) and the need to control pathogens and diseases (Larkin and Griffin 2007), crop rotation is an environmentally effective way for mitigating soil health problems such as soil borne diseases, reduction in crop quality and yield. As reported by Larkin and Griffin (2007), crop rotation is able to control different pathogens and diseases, and reduce soil borne potato diseases (Larkin and Griffin, 2007). A study conducted by Steinberga et al. (2012), evaluating the effect of buckwheat and potato as the fore crops in crop rotation, demonstrated that crop rotation enhances soil quality and microbial diversity (Steinberga et al., 2012). Moreover, buckwheat is considered as an important crop due to its ability to decrease the distribution of plant diseases, is known as a natural suppressor (Steinberga et al., 2012; Rancâne et al., 2009) and it supports higher microbial activity (Steinberga et al., 2012). All cover crops are well known for their ability to improve soil health by physically protecting the soil, increasing organic matter levels, providing biologically fixed nitrogen, enhancing soil nutrients controlling pests (Larkin and Griffin, 2007; Fernandez et al., 2016; Clark et al., 2007). For example, *Brassica* crops are considered as pest management crops due to the fact that they release chemical compounds called glucosinolates, which breakdown to produce isothiocyanates (Larkin and Griffin, 2007; He et al., 2012). Isothiocyanates are toxic to many soil organisms and are considered as bio-fumigants. Thus, they have been used to reduce soil borne populations of fungal pathogens, nematodes, weeds and to enhance soil characteristics and crop yield (Larkin and Griffin, 2007; Clark et al., 2007) Cohen et al., 2005). For example, mustards usually have higher concentrations of isothiocyanates and are known for their allelopathic effects (Clark et al., 2007). There are other mechanisms that are effective in reducing soil borne diseases by *Brassica* crops. These involve *Brassica napus* seed meal that suppresses *Rhizoctonia solani* and this activity is related to particular changes in microbial diversity in the soil, and not the levels of glucosinolate (Larkin and Griffin, 2007; Cohen et al., 2005). Barley is another cover crop that improves soil health, protects crops during drought, produces biomass in a short amount of time, and releases allopathic chemicals that shade out weeds and suppress them (Larkin and Griffin, 2007, Clark et al., 2007). According to Larkin and Griffin (2007), even

though barley does not produce glucosinolates or other volatile antifungal compounds similar to *Brassica* crops, barley had been shown to reduce *Rhizoctonia* diseases in the field (Larkin and Griffin, 2007; Larkin and Honeycutt, 2002; 2006). In their study, these authors demonstrated that barley reduced the amount of inoculum of *R. solani* in a greenhouse setting. In addition, they reported that glucosinolate levels and bio-fumigation are not the only factors that suppress soil borne diseases, but other factors such as the alteration of soil microbial communities, role of the stimulation of soil microbial activity, and volatile compounds and specific isothiocyanate products, might play an effective role as suppressor of soil borne diseases (Larkin and Griffin, 2007). Whereas buckwheat has been used as rotation crop, there are no metagenomic studies reporting its influence on soil microbial diversity. The objective of this chapter is to determine the microbial diversity in the rhizosphere of buckwheat compared with the rhizosphere of other rotation crops.

2.2 Materials and Methods

2.2.1 Field site descriptions

The fields used for this study consisted of one field with high wireworm infestation, located in Stratford (PEI), referred to as Cross Road, and one field with low wireworm infestation located at Harrington farm (PEI), referred to as Harrington. Both fields consisted of red sandy soils. The two sites were about 30 km apart from each other. Before planting in the first year, the soil pH and wireworm densities were determined, as they were also recorded in the subsequent years. Harrington farm is under a 3-year crop rotation program. Therefore, the same crop could not be grown in the same field for two consecutive years contrary to the field at Cross Road location.

2.2.2 Plant materials

Three crop species, namely common buckwheat (*Phagopyrum esculentum*), brown mustard (*Brassica juncea*) and barley (*Hordeum vulgare*) were grown at the two locations in PEI

during 2014 and 2015 growing seasons. At the Harrington location, buckwheat, barley and brown mustard were each grown in 100 m x 100 m side by side plots (in the same field at Harrington farm in 2014). At Cross Road, only buckwheat and barley were each grown in 200 m x 200 m side by side plots during the 2014 and 2015 growing seasons. At this site, the same crop was grown in the same plots in the two consecutive growing seasons (Figure 5).

2.2.3 Soil sampling

2.2.3.1 Soil and plant sampling in the field

A random and systematic sampling strategy was adopted for each plot (Figure 3) as previously suggested (Carter and Gregorich, 2007; Dinkins and Jones, 2008). The strategy consisted of sampling at 5 sites in each plot, with one sampling site in the middle of the plot and 4 sites at each of the four corners of the plots, separated by a 5 meter buffer zone from each border (Figure 5). At each sampling site, 3 samples were collected during each growing season, over two growing seasons. The soil samples at each site were obtained by pulling the plants, ensuring soil was adhering to the roots, and immediately transferred into Ziploc bags. Thus, a total of 15 samples were collected in each plot, for a total of 105 samples over the two growing seasons at the two locations (60 samples from Cross Road and 45 from Harrington). The samples were brought back to the laboratory for further processing (Figure A1, Appendix 1).

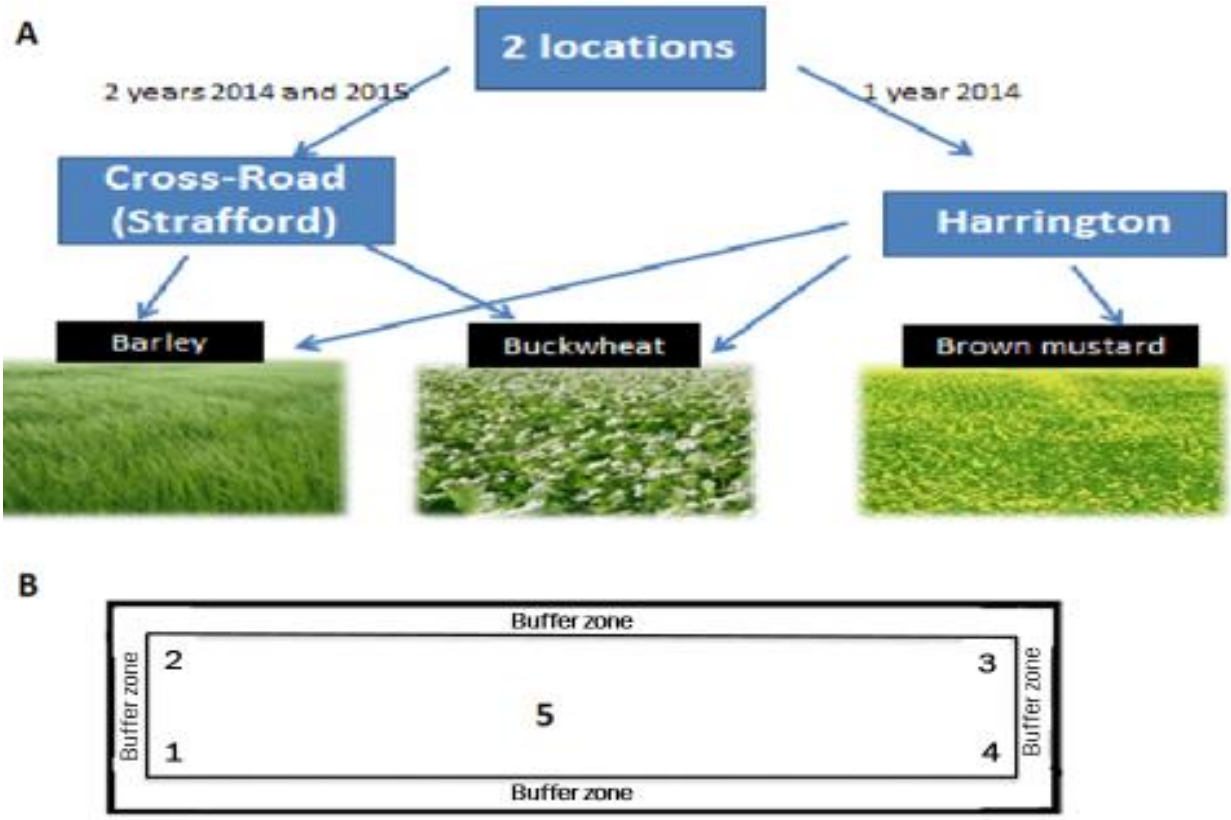

Figure 5. Schematic representation of the study design and sampling strategy. (A) Study design with two locations and 2-3 rotation crops per location. (B) The sampling strategy showing the sampling locations in each plot.

2.2.3.2 Bulk and Rhizosphere soils sampling

In the laboratory, the roots were cut from the stems using scissors and excess of loose soil not sticking to the roots, referred to as bulk soil, was collected in a new bag by gentle shaking. Then, the roots were shaken vigorously to separate as much bulk soil as possible from the roots and the roots were cut and transferred to 15 ml tubes and vortexed to collect root adhering soil, referred to as rhizosphere soil as described by Li et al. (2016). Therefore, two soil samples (bulk

and rhizosphere soils) were obtained from each of the 105 samples collected in the field for a total of 210 soil samples for DNA extraction .

2.2.3.3 Soil DNA extraction

DNA was extracted from soils using MO BIO Power Soil DNA Extraction kit (QIAGEN, Mississauga, ON, Canada) (Table A1, Appendix 1) following the manufacturer's instructions. Briefly, 0.25 gram of soil was added to the provided Power bead tubes and vortexed gently to mix. Solution C1 (60 µL) was added to the mixture and homogenized by vortexing at maximum speed for 10 minutes. After centrifugation at 10,000 *g* for 30 seconds at room temperature, the supernatant (400–500 µL) was transferred to a new 2 mL collection tube and 250 µL of solution C2 was added, and the tube vortexed for 5 seconds, incubated at 4 °C for 5 minutes and centrifuged at room temperature for 1 minute at 10,000 *g*. The supernatant (~600 µL) was collected, transferred to a clean 2 mL collection tube, and 200 µL of solution C3 was added. After briefly vortexing and incubating at 4°C for 5 minutes, the sample was centrifuged for 1 minute at 10,000 *g*. Next, 1200 µL of solution C4 was added to the supernatant (~750 µL) in a new 2 mL collection tube and vortexed for 5 seconds. Approximately 675 µL of the mixture was loaded onto a spin filter column and centrifuged at 10,000 *g* for 1 min at room temperature. The rest of the mixture was loaded onto the same column and centrifuged again. Solution C5 (500 µL) was added to the column and centrifuged for 30 seconds at 10,000 *g* to washing the column. DNA was eluted from the spin filter into new 2 mL collection tubes using 100 µL of solution C6 consisting of sterile DNA-Free PCR Grade Water and centrifuged at room temperature for 30 seconds at 10,000 *g*. DNA samples were further purified according to the manufacturer's procedure and stored at‑80°C. Genomic DNA quality was checked on 1% agarose gel and quantified using the Qubit dsDNA HS assay kit and Qubit Fluorometer (ThermoFisher Scientific, Carlsbad, USA). Based on the DNA concentration obtained from each soil sample, equal amounts of the five DNA samples from each bulk or rhizosphere soil samples at each sampling site in each plot were pooled, leading to a total of 42 pooled DNA samples (Figure A1, Appendix 1).

2.2.3.4 Amplification of the 16S hypervariable regions

The 16S hypervariable regions of the bacterial 16S rRNA were amplified using the Ion 16S Metagenomics Kit™ (Thermo Fisher scientific, Carlsbad, USA) following the manufacturer's recommendations. To increase the resolving power of 16S rRNA profiling, the two sets of primers (V2 and V3) (Figure 4) were obtained from Thermofisher and the primer pool sets were designed to target >80% of sequences found in the Greengenes database (DeSantis et al. 2006) with 100% identity for a primer pair amplifying at least one variable region. The V2 set was used to amplify the variable regions 2, 4, and 8 using the V2-4-8 primer set in a single tube and targeting the amplicon fragments of ~250, 288, and 295 bp respectively. Similarly, the V3 set of V3-6-7-9 primer set from a second single tube was used in a multiplex PCR reaction to target variable regions 3, 6-7, and 9 and amplify the fragments of ~215, 260, and 209 bp, respectively. Briefly, 100 ng of genomic DNA was used for each PCR reaction whereas 1:20 diluted *E. coli* DNA control stock (1.5ng/µL) was used as a positive control. For each sample, two reactions were prepared (one for each of the two primer sets V2-4-8 (V2) and V3-6, 7–9 (V3). One positive and negative control (no template DNA) was also included per PCR run. Thirty µL of PCR mix was prepared and consisted of 15 µL of 2X Environmental Master Mix, 3 µL of 10X 16S Primer Set and 2–12 µL of template DNA sample or 2 µL diluted control. The PCR cycle consisted of an initial denaturation phase at 95°C for 10 min, followed by 25 cycles (18 cycles for positive control DNA) including a denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and an extension at 72°C for 20 sec, and with a final extension at 72°C for 7 min. The presence of PCR products was confirmed by running the reaction product on the Experion™ Automated Electrophoresis System instrument (Bio-Rad laboratories, USA) using the Experion DNA 12K Analysis Kit (Bio-Rad laboratories, USA). An equal volume (20 µL) of V2 and V3 PCR products were pooled for purification using Agencourt AMPure XP Reagent. Agencourt AMPure XP Reagent (1.8X, 72 µL) was added to 40 µL of the pooled V2/V3 amplification reaction. The mixture was briefly vortexed, pulse-centrifuged, and then incubated at room temperature for 5 min. The tubes were placed in a DynaMag-2 magnetic rack for 3 min or until the solution became clear and the supernatant were carefully removed without disturbing the bead pellet. The pellets were washed with 300 µL of freshly prepared 70% ethanol, incubated for 30 sec while turning the tubes around twice in the magnet to move the beads around. After clearing the solution, the supernatant was discarded without disturbing the pellet. The washing step was repeated once again and the remaining supernatant was removed using a 20-µL pipette without disturbing the pellet.

Keeping the tube on the magnetic rack, the beads were air-dried at room temperature for 4 min and 15 µL of nuclease-free water was added directly to the pellet to disperse the beads, vortexed for 5–10 sec, pulse-spin and placed in the magnetic rack for at least 1 min until the solution clarified. The supernatant containing the eluted DNA was transferred to a new 1.5-mL Eppendorf LoBind® Tube without disturbing the pellet. Each purified PCR product (1µL) was analyzed using the Bio-Rad High Sensitivity 12K DNA Kit and an Experion Bioanalyzer® instrument using the Bio-Rad software to determine the amount (ng) of target amplicons.

2.2.3.5 Library preparation

The pooled V2/V3 PCR amplification products were used to prepare a library using the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific) following the supplier's protocols. Briefly, the 100 µL end repair reaction consisted of 79 µL of the purified pooled short amplicons (≈100 ng), 20 µL 5X End Repair Buffer, and 1 µL of End-Repair Enzyme. The mixture was incubated at room temperature for 20 min and the end-repair reaction was purified using 180 µL the Agencourt AMPure XP Reagent (1.8 × sample volume) following the same procedure as described before. The nick repaired purified amplicons were eluted in 25 µL of Low TE. The nick repaired purified amplicons were then subjected to adapter ligation and barcoding in a 100 µL reaction volume composed of 10 µL of 10X Ligase Buffer, 2 µL of Ion P1 Adapter (barcoded libraries), 2 µL of Ion Xpress™ Barcode adapters (1-48), 2 µL of dNTP Mix, 2 µL of DNA Ligase, 8 µL Nick Repair Polymerase and 49 µL nuclease-free water. The reactions were performed under an initial incubation at 25°C for 15 min followed by a second incubation at 72°C for 5 min. The adapter-ligated barcoded amplicons were purified using 140 µL Argencourt AMPure XP Reagent (1.4 × sample volume) following the same procedure as described in the section above. The final purified products were eluted in 20 µL of Low TE.

2.2.3.6 Library quantification using qPCR

To quantify the libraries, four sequential serial 10-fold dilutions were prepared from the *E. coli* DH10B Ion Control Library (68 pM) kit from 6.8 pM to 0.0068 pM and used as a standard. Ten-fold serial dilutions of the sample libraries were prepared from 1:10 to 1:10,000 to target a concentration within the serial dilutions of the control library. Only two dilutions, 1:1000 and 1:10,000 were used for quantitation. qPCR master mix was prepared by mixing 10 µL of TaqMan Fast Universal PCR Master Mix, 1 µL of Ion Library TaqMan Quantitation Assay (20X) and 5 µL of diluted control or sample library (1:1000 or 1:10000 diluted). All reactions were performed in triplicate on the CFX96 real time PCR machine (Bio-Rad) using the FAM dye TaqMan probe reporter/quencher and ROX as a passive reference dye. The serial dilutions of the control library were used as standards. The PCR cycling parameters consisted of an initial hold at 50°C for 2 min, an initial denaturation at 95°C for 20 s, 40 cycles of denaturation at 95°C for 3 s followed by an annealing and an extension at 60°C for 30 s. The diluted library concentration was calculated using real-time PCR instrument software. This information was then used to calculate the undiluted library concentration, and the library dilution factor for template preparation. If both dilutions of the sample library (1:1000 and 1:10000) fell within the standard curve, the undiluted library concentration was calculated for each dilution. Then the average of the two undiluted concentrations was used for final calculation. Otherwise, the undiluted library concentration of the dilution that fell within the standard was used for calculation. The library was finally diluted to 100 pM for downstream template preparation using low TE.

2.2.3.7 Template preparation and sequencing

Template preparation for sequencing was done using Ion PGM™ Template OT2 400 Kit (Cat. no. 4479878, Thermo Fisher Scientific). The amplified libraries were subjected to emulsion PCR using an Ion One Touch 2 system and the Ion PGM Template OT2 400 Ion Sphere Particles Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Briefly, 25 µL of diluted (25 pM final concentration) library was prepared by adding 6.5 µL of the 100 pM library to 18.5 µL nuclease free water. The diluted library was vortexed for 5 s and then centrifuged for 2 s. In a separate tube, 900 µL of reaction mix was prepared by adding 500 µL Ion PGM™ Template OT2 400 Reagent Mix, 285 µL of Ion PGM™ Template OT2 400 PCR Reagent B, 50 µL Ion

PGM™ Template OT2 400 Enzyme Mix, 40 µL of Ion PGM™ Template OT2 400 Reagent X and 25 µL of diluted library. The solution was vortexed at maximum speed for 5 s, then centrifuged for 2 s. One hundred µL of Ion PGM™ Template OT2 400 Ion Sphere™ Particles (ISPs) was added to the 900 µL amplification solution, vortexed at maximum speed for 5 s, and centrifuged for 2 s. The Ion PGM™ OneTouch Plus Reaction Filter Assembly was installed according to manufacturer's instruction and filled with 1000 µL of amplification solution. The amplification reaction was run on the Ion OneTouch™ 2 Instrument for 16 h according to the manufacturer's instruction. The resulting Ion Sphere Particles (ISPs) were enriched using the Ion One Touch ES (Thermo Fisher Scientific). Sequencing was conducted at 850 flows using the Ion PGM™ Sequencing 400 Kit on the Ion Torrent Personal Genome Machine (PGM) (Thermo Fisher Scientific) and 1-16 barcoded samples were pooled and loaded onto 314 or 316 Chips (Figure A2, Appendix 3).

2.2.3.8 Data analysis

After the sequencing runs, all sequencing read data generated were saved under different formats, including BAM files, by the Ion Reporter software Suite V5.0 (ThermoFisher Scientific) which automatically performs read mapping, annotation, and reporting the library and sequencing quality (loading, the total usable reads, mean and median reads length) for each 16s RNA primer region as well as the percentage of the total, valid, and mapped reads for each taxonomical level. All unique reads were exported to FASTA files with unique ID to keep track of the analysis results throughout. These files were then passed to a command-line BLAST (E value of 0.01) process and the output data stored in result files in TXT format. Keeping flexible bacterial identification in mind, the 16S rRNA workflow module in Ion Reporter™ Software was designed to classify individual reads by mapping to three reference library options using the BLAST tool to: (i) the curated Greengenes database, containing >400,000 curated records; (ii) to the premium curated MicroSEQ® ID database, a high-quality library of full-length 16S rRNA sequences for >15,000 organisms manually curated for sequence quality, length, annotation, and phylogeny, with frequent taxonomical updates; and (iii) to both reference libraries for access to manually curated and public content and involving an optimal two-step BLAST alignment. After all result

files are generated by the BLAST processes, the algorithm assigns taxonomy information and percent match. If a unique read did not get a pass mark matching result after the BLAST process, the read is marked as LOW-SCORING or UNMAPPED and reanalyzed against the next stage database (DB). The default approach was that the analysis started with the smaller database and then went to the larger one for reads that did not get a good result in the first one. The algorithm supports 1-N stages, not only 1-2. Whenever a read gets a better result in stage-1 DB mapping than in the next, the best result is used. If a read did pass , it was marked and not analyzed again in the next stage. After all reads were checked against stage-1 DB, the UNMAPPED and LOW-SCORING reads were checked against stage-2 DB and so on. After the last stage, any read that did not map at all was exported to an unmapped reads FASTA file.

Taxonomic assignments were reported as a consensus of the results from all of the primers and by each primer, with the option to report multiple taxonomical assignments (slash call). Slash calls can result for a particular variable region when a sequence identifies multiple taxa within a set percentage range. By default, alignment at various taxonomical levels follows the Clinical and Laboratory Standards Institute (CLSI) guidelines requiring the family level to have <97% identity, with genus >97% identity and species >99% identity (Edgar, 2017). It is important to mention that below the genus level identification, taxonomic assignment should be considered presumptive family level. Family assignment was the consequence of an assignment to the best match, but this assignment might not capture other nearly equivalent assignments that could be equally valid. Biological analysis and identification of microbes was enhanced through interactive graphs (powered by Krona) (Ondov et al., 2011) to enable data exploration with visualization of results by primer or consensus that can be graphically viewed at six taxonomic ranks (species, genus, family, order, class, phylum). The 16S metagenomics workflow in Ion reporter v 5.0 reported the alpha diversity analysis and beta diversity analysis using the QIIME (Quantitative Insights In to Microbial Ecology) (Edgar, 2017). The results were further downloaded to the computer and exported to Microsoft Excel file to calculate the percentage of each species identified in each crop and each field (bulk and rhizosphere), and a heat map was generated to display the difference in terms of abundance between the samples based on their respective OTU abundances. It is important to note that since Brown mustard was grown only at Harrington location in 2014, it was removed from the rest study and focus only on buckwheat and barley. Further statistical analyses (ANOVA) were performed using SAS and GENSAT packages to display var-

iations in bacterial diversity variations that were found between the buckwheat and the barley's bulk and rhizosphere soils.

The microbial diversity data were exported into Excel in the form of count per OTU in each sample and an analysis of variance (ANOVA) was further performed using GENSAT 64 bit v18.1 with OTUs, crops, locations, the soil types (bulk or rhizosphere), years, taxonomic levels (family, genus. species), and replications as factors, and counts as variables. In addition, an ANOVA was performed using frequency in SAS Studio 3.71 (University Edition).

2.3 Results

2.3.1 Sequencing depth and accuracy in data acquisition

The observed total number of reads varies from run to run and between chip types used for the sequencing (314-chip, 316-chip). In this study, the total number of reads per run ranged from 375,974 to 709,961 reads using the 314 chip holding two libraries in each run, and from 1,869,147 to 3,343,355 reads using the 316 chip holding 4 -7 libraries in individual runs. The total number of reads in all libraries sequenced ranged from 145,144 to 1,247,112. The number of mapped reads per sample varied from 14,937 to 187,461 (Table A2, Appendix 2). In addition, the mean read length ranged from 134 to 194 bp for both chips. In the Ion torrent suite software.

2.3.2 Alpha diversity

Sequencing at sufficient depth is crucial to fully describe bacterial diversity (Zaheer et al., 2018). A rarefaction analysis that plots the number of taxa as a function of sequences per sample was performed using Alpha Diversity tools. Rarefaction analysis of these indices (for example, index value as a function of sequencing depth) was also performed and is shown in Figure 6. Alpha diversity estimates the number of taxa (richness) and distribution (evenness) within a single microbial ecosystem using different metrics such as the Chao1, Shannon, and Simpson indices (Morgan and Huttenhower, 2012; Goodrich et al., 2014; Kuczynski et al., 2010; Lozupone and Knight, 2008). To compare the diversity between sites, several diversity indices were

calculated (Chao1, Shannon, and Simpson index). In the current study, the sequencing results showed that microbial richness increased in all samples up to 299 sequences/sample and reached a plateau after ~300 sequences in each sample (Figure 6 and 7), indicating that sequencing was performed to a sufficient depth. The microbiome diversity estimated in terms of alpha diversity in buckwheat was found to be higher in the rhizosphere soil samples compared to the bulk soil, independent of the growing year (Figure 6 A – D). For barley and buckwheat, a good correlation was observed between the Chao1, observed species, as well as Shannon and Simpson estimators for the alpha diversity (Figure 6 and 7).

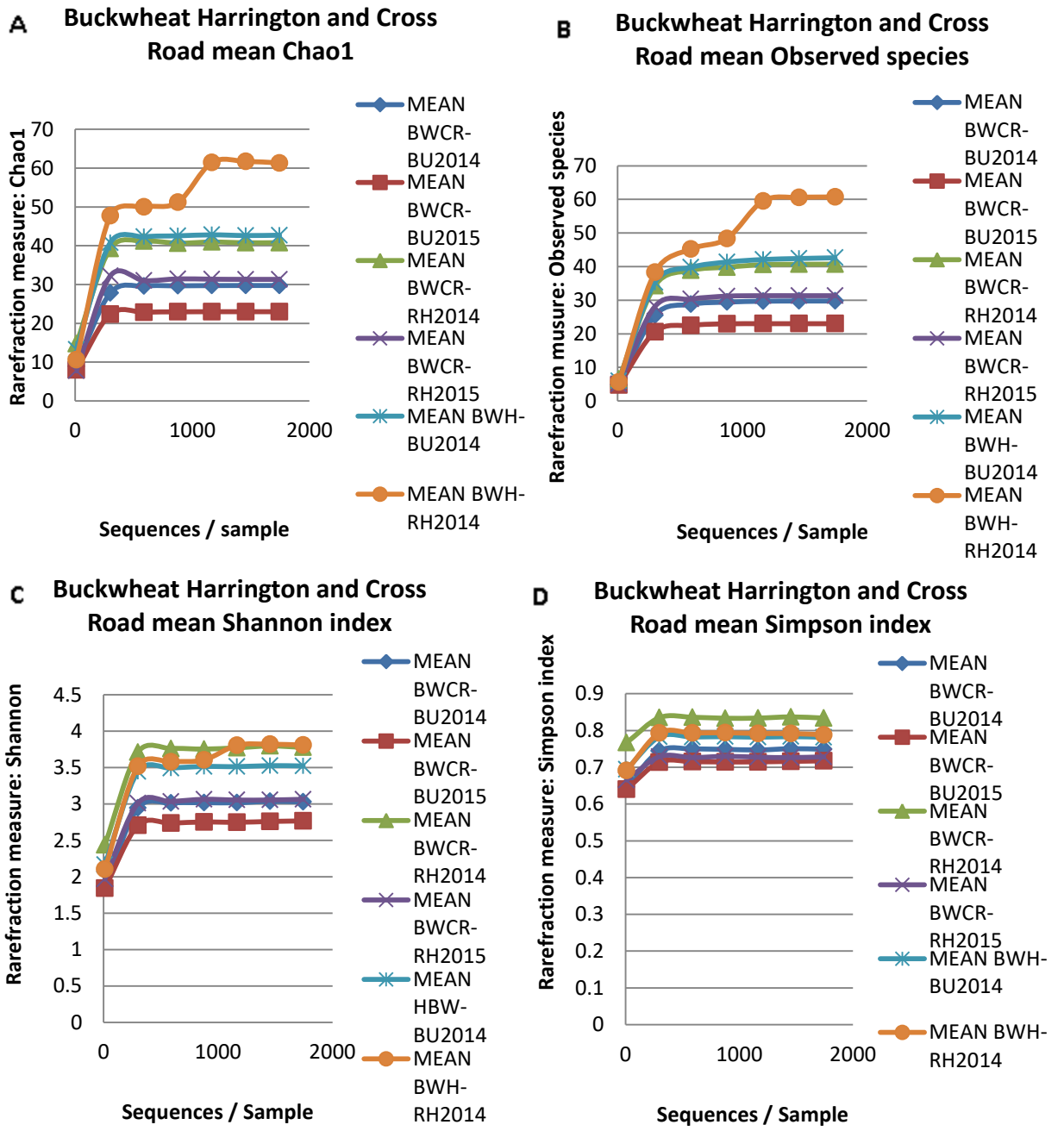

Figure 6. Rarefaction curves of Alpha diversity in buckwheat at Harrington and Cross Road locations (represents the richness of the sample according to the number of sequences). The Alpha diversity was estimated using: A) Chao1, which estimates the number of species (richness and evenness); B) Observed species; C) Shannon index estimates of diversity (taking in account the number of species and how evenly they are distributed; D) Simpson index. BW, buckwheat; CR, Cross Road; H, Harrington; BU, bulk; RH, rhizosphere.

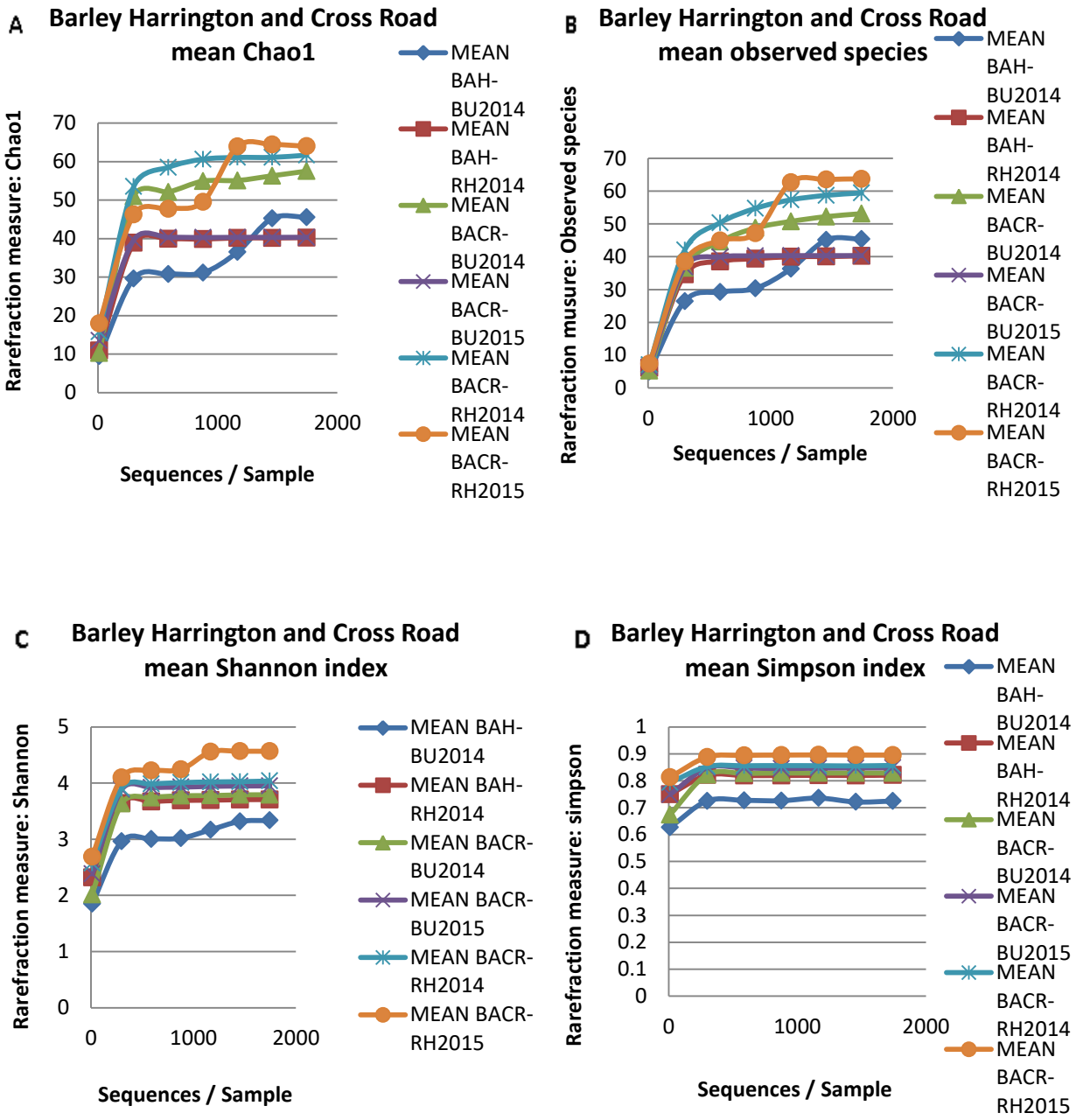

Figure 7. Rarefaction curve of Alpha diversity in barley at Harrington and Cross Road locations. The Alpha diversity was estimated using A) Chao1, estimates the number of species in the sample; B) Observed species; C) Shannon index estimates the diversity and takes in account the number of species and defines how even they are distributed; D) Simpson index. BA, barley; CR, Cross Road; H, Harrington; BU, bulk; RH, rhizosphere.

2.3.3 Beta diversity

Beta diversity describes and compares the spatial variation in the composition of microbial communities between samples at the species level and provides a measure of the distance, similarity/dissimilarity, or relative abundance of taxonomic units between samples. Principal coordinates analysis (PCoA) was used to visualize the data of the beta diversity distance matrix in the form of 2 or 3- Dimensional plots known as PCoA plots (Goodrich et al., 2014). PCoA transforms the distance matrix into a new set of orthogonal axes called principle components (PC). In this study, PC1 explained the maximum amount of variation found in the dataset (80.28%), followed by PC2 (4.66%) and PC3 (2.84%). In the PCoA plot below (Figure 8) each dot represents an individual sample (which was replicated), and the points that are close to each other represent the similarity in the microbial composition or the community composition of these samples that appear to be related and close to one another (Figure 8). However, the distance between points represents how the samples are compositionally different from one another as can be seen for BACR2014RH2 (barley Cross Road rhizosphere), BACR2014BU3 (barley Cross Road bulk), and BWHRH2 (buckwheat Harrington rhizosphere) (Figure 8 A and C), and this variation is related to sequencing and valid reads per sample.

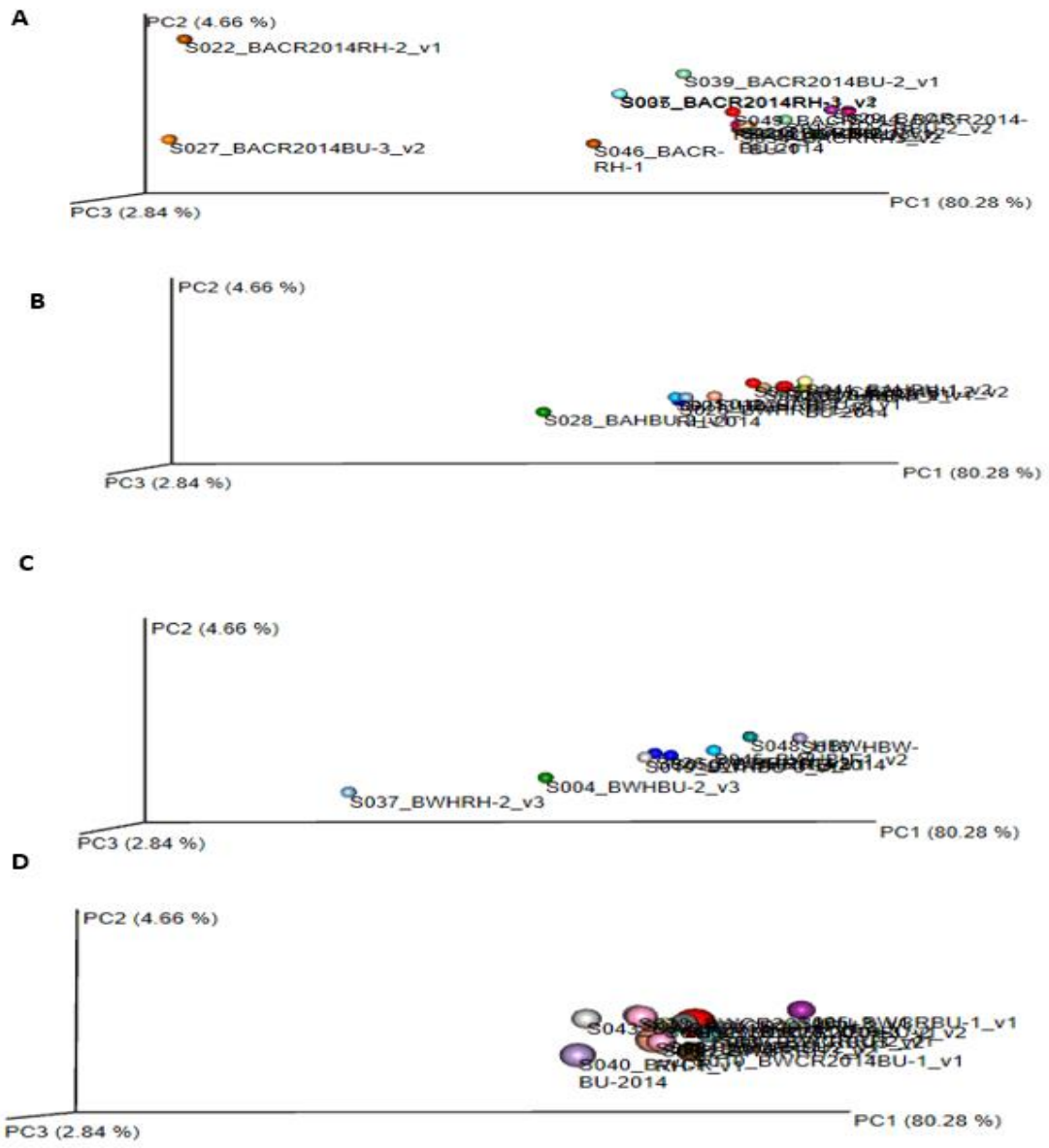

Figure 8. Principal Coordinates Analysis (PCoA) showing the variation in the composition of microbial communities between samples at species level. A) barley Cross Road (bulk & rhizosphere soil), B) barley Harrington (bulk & rhizosphere soil), C) buckwheat Harrington (bulk & rhizosphere soil), D) buckwheat Cross Road (bulk & rhizosphere soil). In principal coordinates analysis, each point represents a replicate, and the points that are closer to one another are more similar in terms of microbial community.

2.3.4 Analysis of microbial diversity

2.3.4.1 Microbial diversity in terms of frequency at each taxonomy level

An analysis of microbial diversity was performed for individual samples and the different taxonomic levels were inferred for each primer set (Figure 9). A total of 27 phyla were observed in both the bulk and rhizosphere soil at Harrington and Cross-Road locations (Figure 10). Variations in microbial composition and abundance were observed between bulk and rhizosphere soils in each crop at each location. Variations in microbial diversity were also observed between crops within and between locations. *Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Deinococcus-Thermus* and *Crenarchaeota* (Figure 11) were the 7 phyla for which confident identifications up to species level were achieved. *Proteobacteria* was found to be the most dominant phylum in the study (Figure 10, 11).

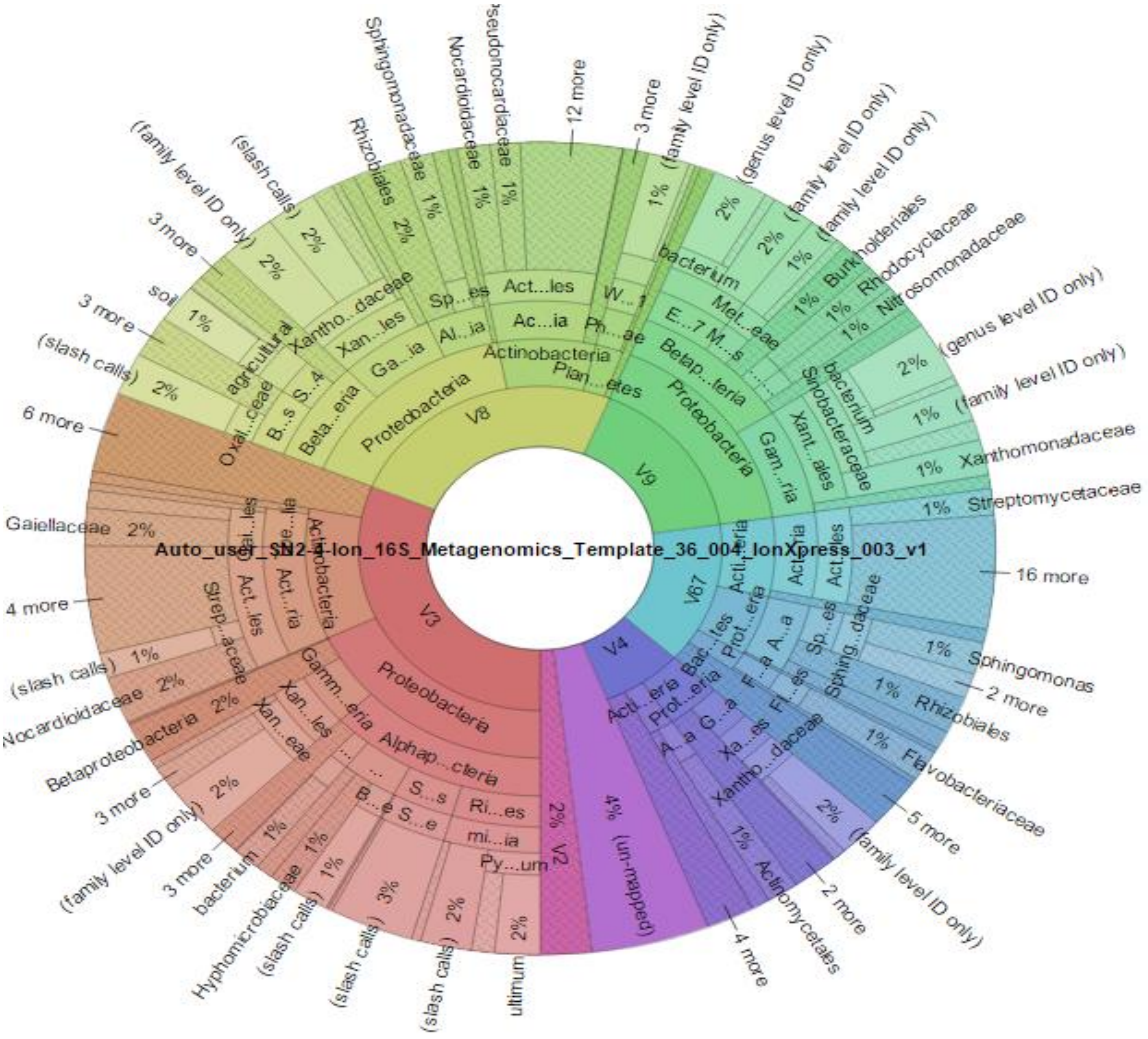

Figure 9. An example of Krona diagram generated by the Ion Torrent software showing the taxonomic composition of microorganisms as identified by different primer sets (V3-6,7-9).

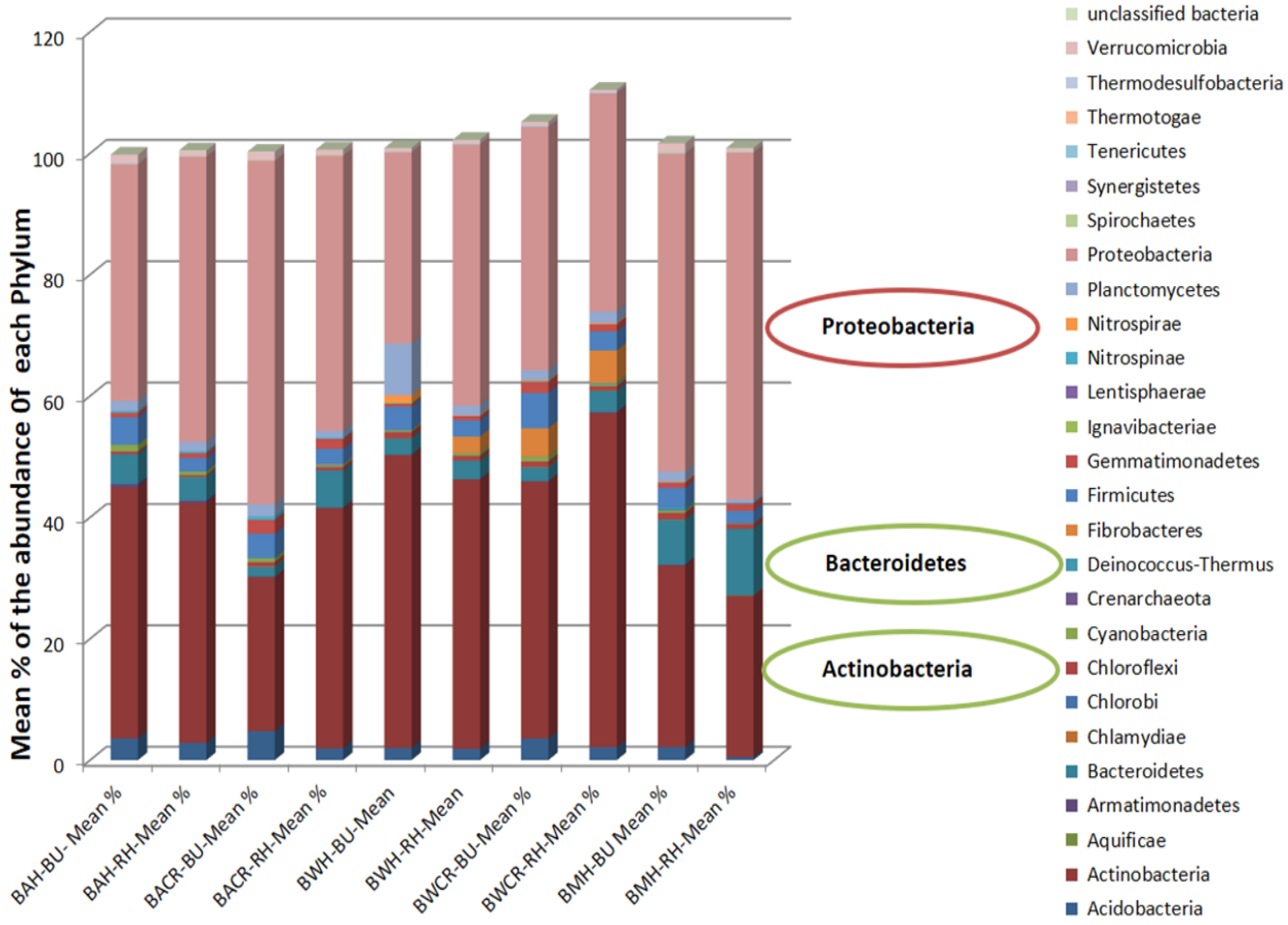

Figure 10. The distribution of the 27 different phyla identified in the bulk and rhizosphere soil samples from Harrington and Crossroad (Stratford). The three most abundant phyla are indicated.

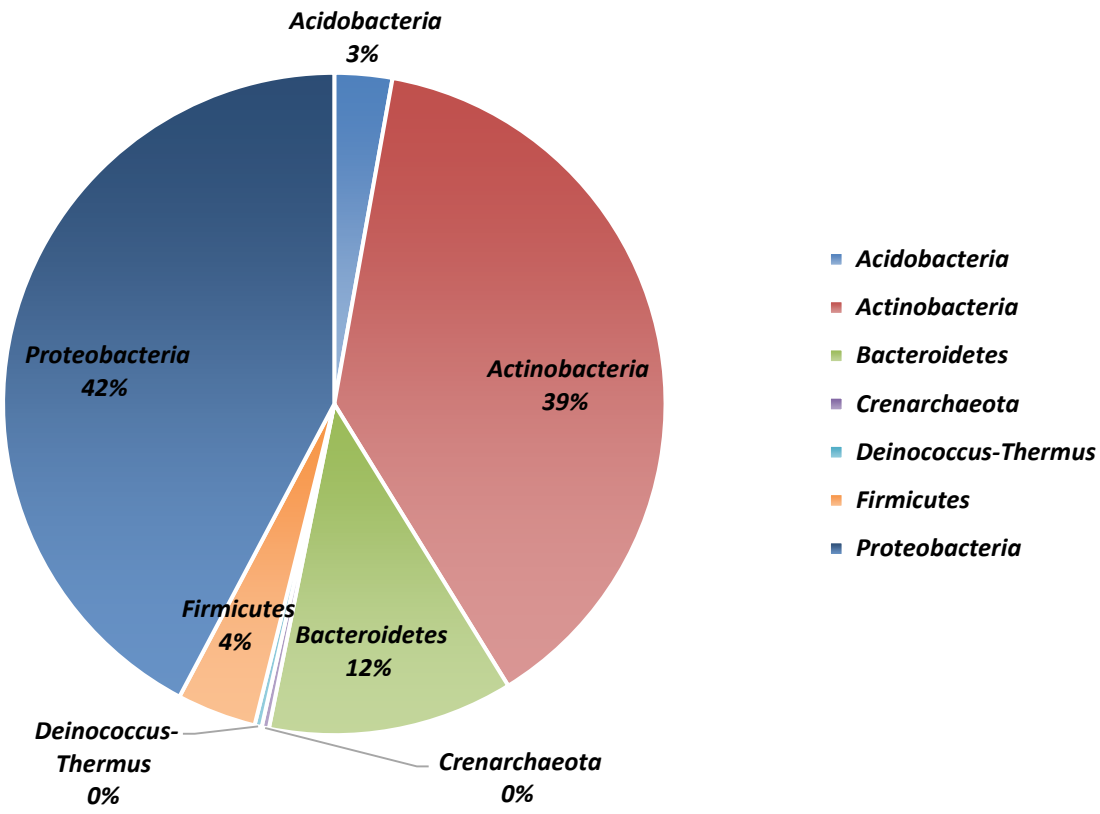

Figure 11. The distribution of 7 phyla identified up to species level, illustrating their relative percentages.

Since there was no difference in term of richness at the higher (phylum, order, and class) levels, an ANOVA analysis was performed at the family, genus and species levels using the observed OTU counts and frequencies as variables.

2.3.4.1.1 Microbial diversity at the family level

Using frequency (counts) as variables in SAS, more OTUs were observed at the family level in the barley bulk soil (153±40) at the Cross Road location in 2014 in comparison with the buckwheat bulk soil (134±24) at the same location. However, the difference was not statistically significant (P = 0.44). In the rhizosphere soil, more OTUs were observed, at statistically signifi-

cant higher levels (p = 0.05) in the barley compared to the buckwheat. In 2015, more OTUs were identified in the bulk soil of barley bulk at Cross Road compared with the bulk soil of buckwheat (p < 0.05). In the same year, although, more OTUs were found in the rhizosphere soil from barley at Cross Road compared with the rhizosphere soil from buckwheat; the difference was not statistically significant (p = 0.33) (Figure 12; Table 3).

At the Harrington location in 2014, barley also showed more OTUs in the bulk soil than that collected from buckwheat but the difference was not significant (p = 0.2). Similarly, no significant difference (p = 0.24) was observed between rhizospheres from barley and buckwheat at the family level (Table 3).

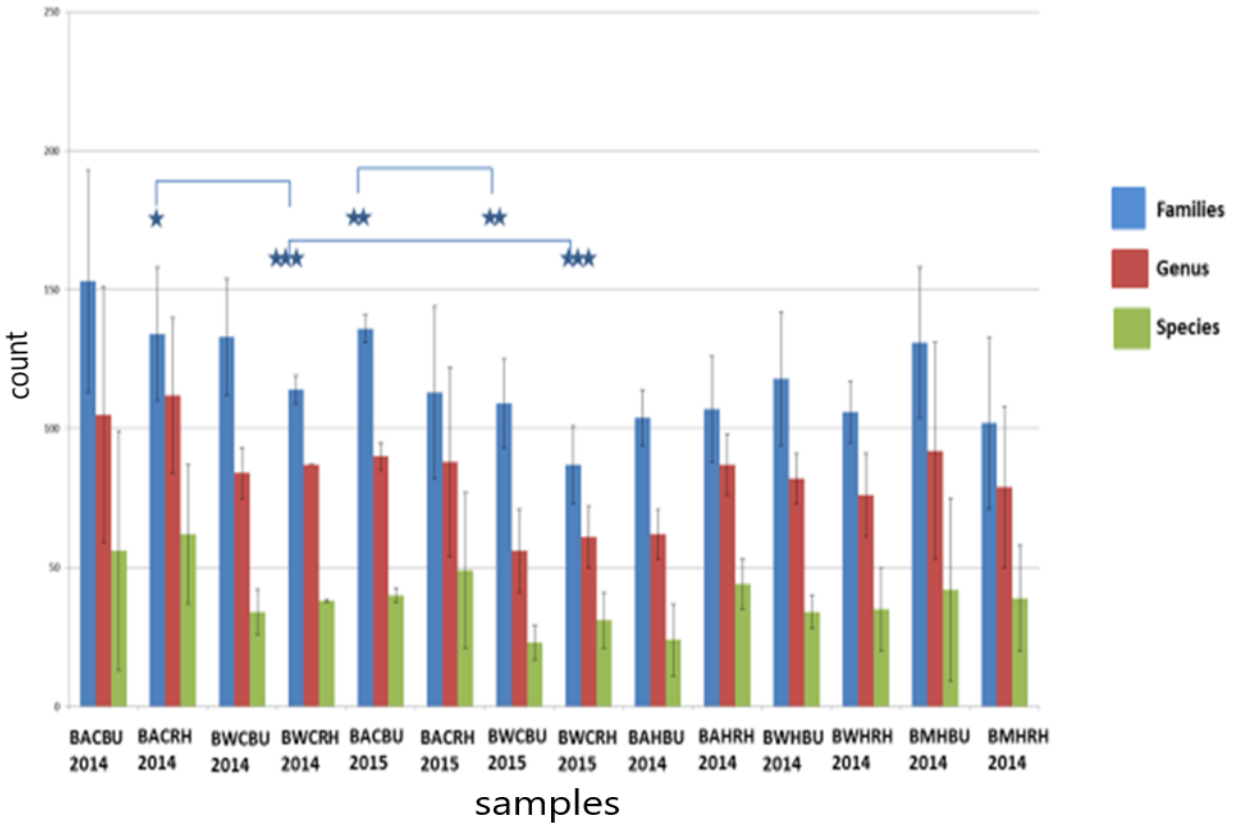

Figure 12. Microbial diversity observed at different taxonomic levels in different soil types (bulk versus rhizosphere) in barley and buckwheat over 2 growing seasons (2014 and 2015). C, Cross-road; H, Harrington; BA, barley; BW, buckwheat; Bu, Bulk soil; RH, rhizosphere. Stars indicate levels of statistical significant difference at the family level.

Table 3. ANOVA statistics of microbial diversity at the family taxonomic unit level in the soils collected from buckwheat and barley at Cross Road and Harrington locations in 2014 and 2015.

year	Location	Crop	Soil type	P value
2014	Cross Road	Barley / Buckwheat	Bulk	0.4
2014	Cross Road	Barley / Buckwheat	Rhizosphere	0.05
2015	Cross Road	Barley / Buckwheat	Bulk	< 0.05
2015	Cross Road	Barley / Buckwheat	Rhizosphere	0.33
2014	Harrington	Barley / Buckwheat	Bulk	0.2
2014	Harrington	Barley / Buckwheat	Rhizosphere	0.24

2.3.4.1.2 Microbial diversity at the genus level

At the genus level and using frequency (counts) as variable, more OTUs were observed in the bulk soil from barley at the Cross Road location in 2014 than that collected from buckwheat at the same location, but the ANOVA did not show a significant difference ($p = 0.5$). Similarly, although a difference was observed between the barley rhizosphere and buckwheat rhizosphere for the number of OTUs, this difference was not statistically significant ($p = 0.1$). In the 2015 growing season at the same location however, a significant difference ($p< 0.05$) was observed between the bulk soil collected from barley and buckwheat. For the rhizosphere soil, even though a higher number of OTUs were observed in barley at Cross Road in comparison with buckwheat at the same location, this difference was not statistically significant ($p = 0.33$) (Table 4).

At the Harrington location in 2014, there was no significant difference ($p = 0.1$) between the bulk soil from barley and buckwheat although a slight difference was observed in the number of OTUs. As previously observed at the family level, no significant difference ($p = 0.3$) was observed between rhizospheres from barley and buckwheat at the genus level (Table 4).

Table 4. ANOVA statistics of microbial diversity at the genus taxonomic unit level in the soils collected from buckwheat and barley at Cross Road and Harrington location in 2014 and 2015.

year	Location	Crop	Soil type	P value
2014	Cross Road	Barley / Buckwheat	Bulk	0.5
2014	Cross Road	Barley / Buckwheat	Rhizosphere	0.1
2015	Cross Road	Barley / Buckwheat	Bulk	< 0.05
2015	Cross Road	Barley / Buckwheat	Rhizosphere	0.33
2014	Harrington	Barley / Buckwheat	Bulk	0.1
2014	Harrington	Barley / Buckwheat	Rhizosphere	0.3

2.3.4.1.3 Microbial diversity at the species level

Microbial diversity was further assessed at the species level in barley and buckwheat at the two locations based on frequency (counts) as variables (Table 5). At the Cross Road location in 2014, although the microbial population count indicated a difference between the bulk soils from barley and buckwheat, the difference was not statistically significant ($p = 0.5$). The same observation was made for the rhizosphere soils from the two crops ($p = 0.1$). However, in 2015 the results showed statistically significant ($p < 0.05$) differences between the bulk soil from barley and buckwheat. Even though the number of OTUs was higher in the rhizosphere from barley compared with buckwheat, the difference was not significant ($p = 0.33$) (Table 5).

Similar to the Cross Road location in 2014, no significant difference ($p = 0.2$) was observed between the bulk soils from barley and buckwheat at the Harrington location. Moreover, there was no statistical difference ($p = 0.4$) (Table 5) between the number of OTUs present in the barley rhizosphere and that of buckwheat at Harrington in 2014.

Table 5. ANOVA statistics of microbial diversity at the species taxonomic unit level in the soils collected from buckwheat and barley at Cross Road and Harrington location in 2014 and 2015.

year	Location	Crop	Soil type	P value
2014	Cross Road	Barley / Buckwheat	Bulk	0.5
2014	Cross Road	Barley / Buckwheat	Rhizosphere	0.1
2015	Cross Road	Barley / Buckwheat	Bulk	< 0.05
2015	Cross Road	Barley / Buckwheat	Rhizosphere	0.33
2014	Harrington	Barley / Buckwheat	Bulk	0.2
2014	Harrington	Barley / Buckwheat	Rhizosphere	0.4

Taken together, a reduction in microbial load was observed over years at Cross Road (Table 6). Buckwheat showed a higher reduction over years compared to barley, with 20.3% and 32% reductions at the family and genus level, respectively compared to barley for which 13.3 and 17.6% reductions were observed for the same taxonomic levels. At the species level, 25.0 % and 25.4 % reductions were observed for buckwheat and barley, respectively (Table 6).

Table 6. Variation of observed OTUs number at the family, genus and species taxonomic levels at Cross Road and Harrington locations in 2014 and 2015. Values shown are means from bulk and rhizosphere soils for each taxonomic level.

Location	OTU level	2014		2015	
		BA	BW	BA	BW
Cross Road	Family	143	123	124	98
	Genus	108	85	89	58
	Species	59	36	44	27
Harrington	Family	105	112	NA	NA
	Genus	75	79	NA	NA
	Species	34	35	NA	NA

NA = Not applicable as no study conducted at Harrington location in 2015. BA = barley, BW = buck-wheat.

2.3.4.2 Microbial diversity in terms of richness

Using counts as variables in GenStat, the microbial diversity at species level was determined. The results indicated that there was a difference ($P<0.05$; $P=0.016$) in terms of richness between Harrington and Cross Road (Stratford). However, there was no significant difference at the species level between 2014 and 2015 ($P = 0.1$). Moreover, a difference ($P<0.05$) was observed between barley and buckwheat, indicating a variation in the microbial richness between these two crops. The variation based on soil type (bulk and rhizosphere) was significant ($P<0.05$; $P = 0.02$) for two crops. A significant difference ($p<0.001$) was observed at the taxonomic (Family, Genus and Species) levels. Although, the interactions between location and crop was significantly different ($P< 0.001$), there was no significant interactions between location and soil type (bulk and rhizosphere) ($P= 0.2$). Whereas the interaction between year and crop was highly significant ($p < 0.001$), the interaction between year and type (bulk and rhizosphere) was not significant ($P = 0.3$). In addition, a significant interaction was observed between year and taxonomic level ($P < 0.05$) (Table 7).

There was no interaction between location, crop, type of soil ($P = 0.6$). Also there was no significant difference ($P = 0.4$) based on year, crop and type. However, there was a significant interaction based on location, crop, taxonomic level ($P < 0.001$) as well as year, crop, taxonomic level ($P = 0.001$) (Table 7).

Table 7. Analysis of variance of microbial diversity in terms of richness.

Source of variation	P- Value

Loc	0.016*
Year	0.1
Crop	0.05*
Type	0.02*
Taxo	< 0.001*
Loc. x Crop	< 0.001*
Year. x Crop	< 0.001*
Loc. Type	0.2
Year. Type	0.3
Crop. Type	0.5
Loc. Taxo	0.1
Year. x Taxo	0.05*
Crop. Taxo	0.1
Type. Taxo	0.4
Loc. Crop. Type	0.6
Year. Crop. Type	0.4
Loc. x Crop. x Taxo	< 0.001*
Year. x Crop. x Taxo	0.001*
Loc. Type. Taxo	0.6
Year. Type. Taxo	0.7
Crop. Type. Taxo	0.9

Loc = Location; Crop = barley & buckwheat; year = 2014 & 2015; Type = bulk & rhizosphere; Taxo = Family, Genus & Species. * indicates a significant value.

2.3.5 Comparison of microbial diversity in 2014 and 2015

In this study, the bacterial populations varied between barley and buckwheat, bulk and rhizosphere and also between Harrington and Cross Road sites. The variation between the microbial populations varied between 2014 and 2015 at the two locations. In 2014, a total of 227 species were identified in barley bulk and rhizosphere at the Cross Road, whereas 127 were identified in the same types of samples at the Harrington. In buckwheat samples in contrast, 201 species were identified in buckwheat at Harrington whereas 138 species were identified in buck-

wheat at Cross Road (Figure 13). In 2015, the total number of species found in barley and buckwheat at Cross Road were 157 and 168, respectively (Figure 13). For each year, a slightly higher number of species was found in the rhizosphere compared to bulk soil samples.

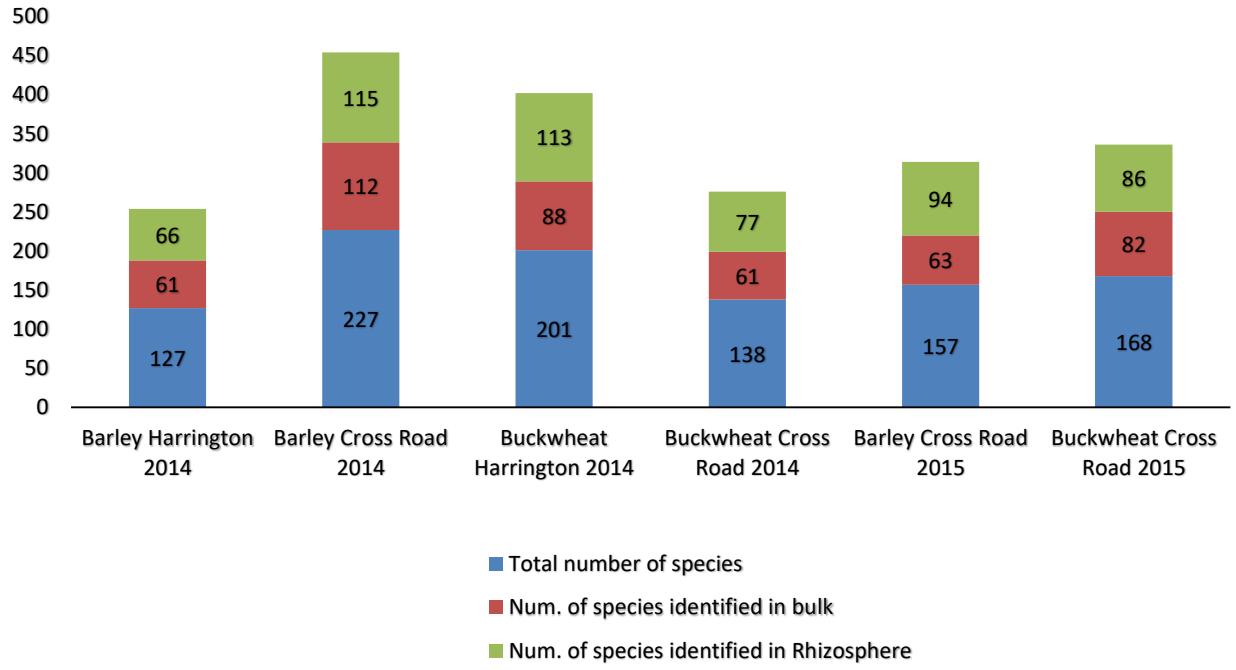

Figure 13. Variations in microbial richness by year. Number of species identified in the bulk and rhizosphere soil samples of buckwheat and barley at the Harrington and Crossroad (Stratford) locations in 2014 and 2015.

2.3.5.1 Microbial diversity in barley rhizosphere soil

In 2014, 115 species-level OTUs were identified in the barley rhizosphere at Cross Road (Figure 14.1). Among these OTUs, 17 were unique to the Barley rhizosphere at this location (Table 8.1). At Harrington, 66 OTUs were found in the barley rhizosphere (Figure 14.1), of which 5 OTUs appeared to be unique to the barley rhizosphere at Harrington (Table 8.1). Interestingly, 4 OTUs were found to be unique to the barley rhizosphere at both the Cross Road and Harrington locations.

In 2015, a total of 94 OTUs were observed in the barley rhizosphere at Cross Road (Figure 14.3), 11 of which were unique to the Barley rhizosphere at Cross Road (Table 8.1).

2.3.5.2 Microbial diversity in barley bulk soil

At the Cross Road location, a total of 112 species-level OTUs were identified in the barley bulk soil in 2014 (Figure 14.1), some of which were common between barley bulk and barley rhizosphere soil, whereas 19 were unique to barley bulk soil at Cross Road (Table 11). At Harrington, 61 OTUs were observed in the barley bulk soil in 2014 (Figure 14.1), of which only 5 OTUs were unique to the barley bulk soil at this location (Table 8.1). In 2015, 63 OTUs were identified in barley bulk soil at the Cross Road location (Figure 14.3) and 4 of them were unique (Table 8).

2.3.5.3 Microbial diversity in the buckwheat rhizosphere soil

In 2014, a total of 113 species-level OTUs were identified in the buckwheat rhizosphere soil at Harrington (Figure 14.2), of which 14 OTUs were found to be unique to the buckwheat rhizosphere soil (Table 8.1). At the Cross Road location in contrast, 77 OTUs were identified in the buckwheat rhizosphere in the same year (Figure 14.2), of which 3 OTUs were unique to the buckwheat rhizosphere. In 2015, 86 OTUs were identified in the buckwheat rhizosphere at the Cross Road location (Figure 14.3), 3 of which were unique to the buckwheat rhizosphere (Table 8.1). In addition, one species (*Streptomyces cacaoi*) was only found in the buckwheat rhizosphere at the Cross Road location, and is considered as a common species for 2014 and 2015 (Table 8.2).

2.3.5.4 Microbial diversity in buckwheat bulk soil

In 2014, 88 species-level OTUs were found in the buckwheat bulk soil at Harrington (Figure 14.2). Three (3) of these OTUs were only present in the bulk soil collected from buckwheat at Harrington (Table 8.1). At Cross Road however, 61 OTUs were identified in the buckwheat bulk soil (Figure 14.2), and 4 were considered as unique (Table 8.1).

In 2015, 82 OTUs were identified in the buckwheat bulk at Cross Road (Figure 14.3), and 3 of these OTUs were unique to this soil type (Table 8.1).

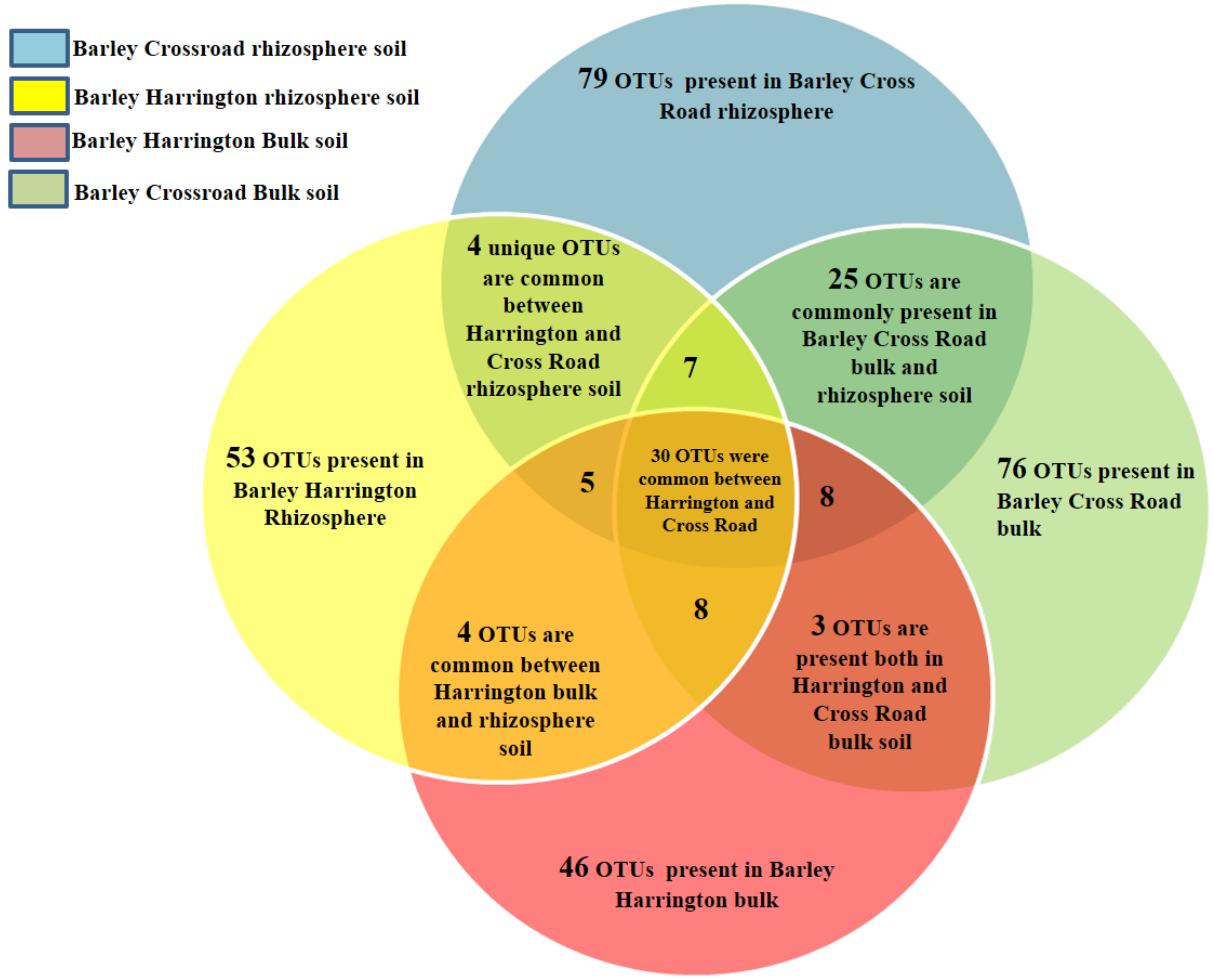

Figure 14.1. Venn diagram showing the distribution of species-level OTUs at the Harrington and Cross Road locations. Number of OTUs identified in the bulk and rhizosphere soils of barley at Cross Road and Harrington in 2014. 6 OTUs were found common between BA CRBU & BA HRH or BA HBU & BA CRRH, and cannot be presented in Figure 14.1. BA, barley; BU, bulk; RH, rhizosphere; CR, Cross Road; HR, Harrington.

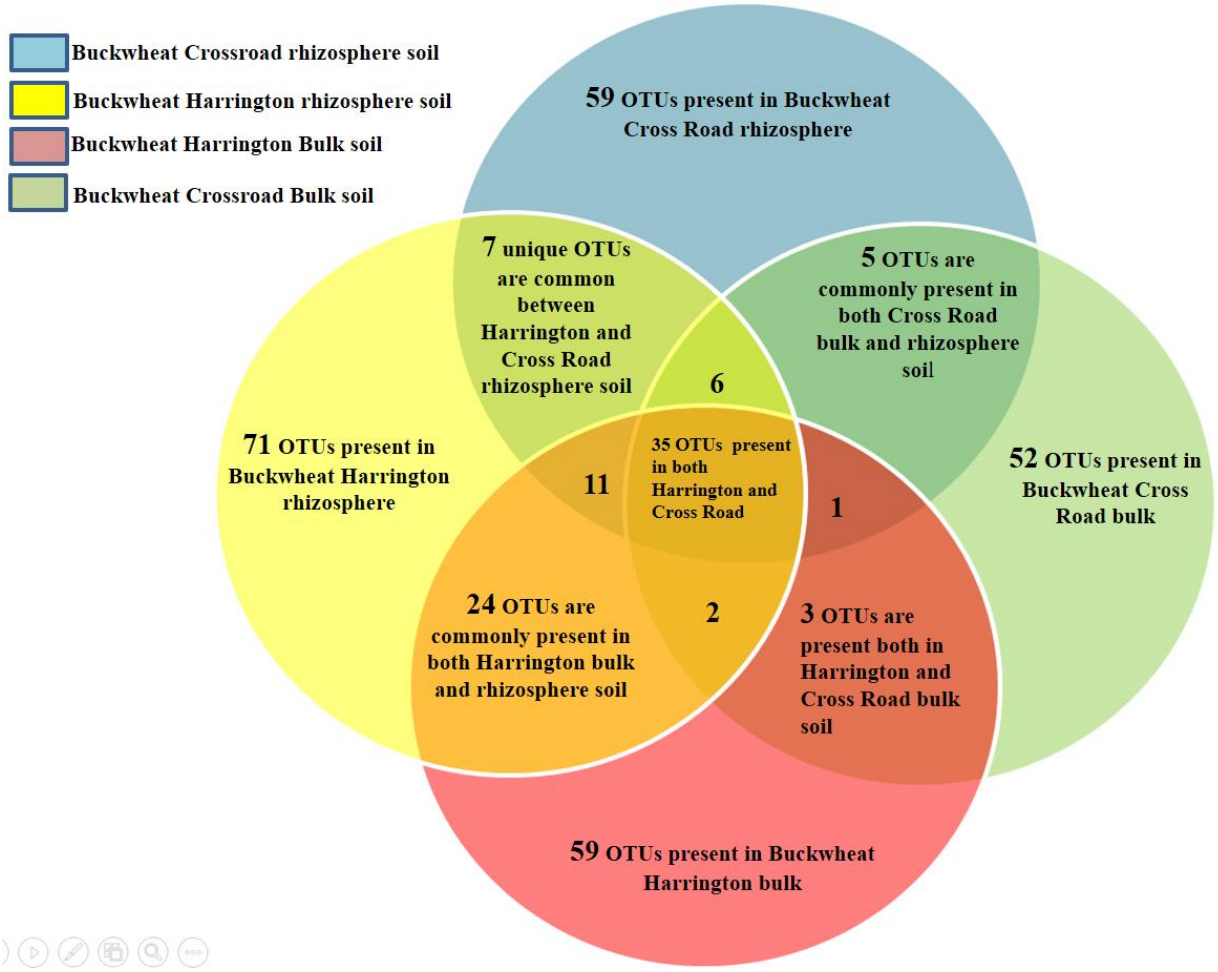

Figure 14.2. Venn diagrams showing the distribution of microbial diversity at the Harrington and Cross Road locations. Number of OTUs identified in the bulk and rhizosphere soils of buckwheat at Cross Road and Harrington in 2014. Four were found common between BW CRBU & BW HRH or BW HBU & BW CRRH, and cannot be presented in Figure 14.2. BW, buckwheat; BU, bulk; RH, rhizosphere; CR, Cross Road; HR, Harrington.

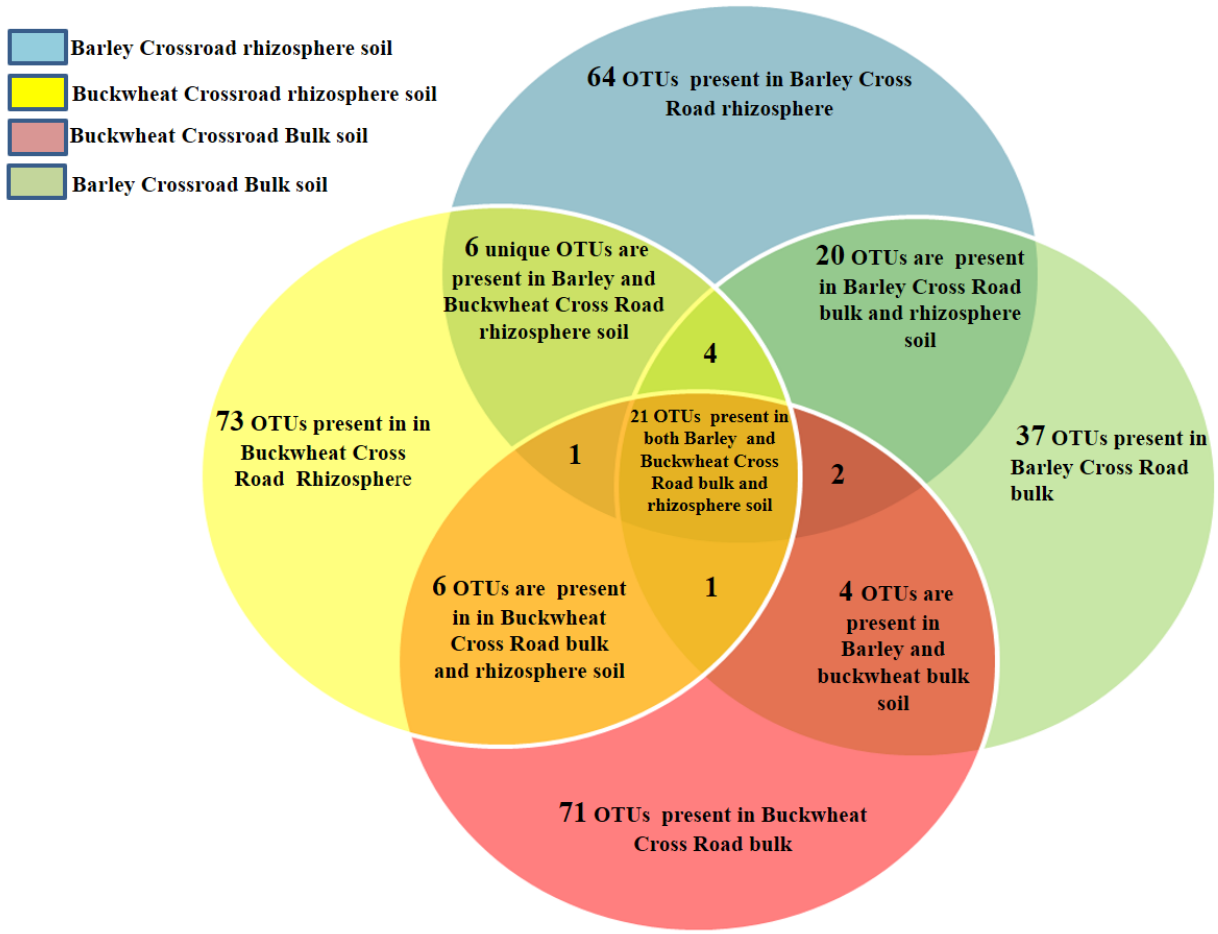

Figure 14.3. Venn diagrams showing the distribution of microbial diversity at the Harrington and Cross Road locations. Overlap of OTUs in 2015 (barley Cross Road and buckwheat Cross Road). 15 OTUs were found common between BW CRBU & BA HRH or BW HBU & BA CRRH, which cannot be presented in Figure 14.3. BA, barley; BW, buckwheat; BU, bulk; RH, rhizosphere; CR, Cross Road; HR, Harrington.

Table 8.1. Unique bacterial species found only in the bulk or rhizosphere soil types from each crop dur-

ing the 2014 and 2015 growing seasons.

Soil Types	2014 bacterial species	2015 bacterial species
BA-CR-BU	*Aciditerrimonas ferrireducens*	*Cupriavidus necator*
	Allokutzneria albata	*Lentzea albida*
	Amycolatopsis saalfeldensis	*Pantoea ananatis*
	Angiococcus disciformis	*Sphingomonas faeni*
	Aquabacterium parvum	
	Archangium gephyra	
	Arthrobacter psychrochitiniphilus	
	Brevundimonas variabilis	
	Clostridium tagluense	
	Flavobacterium terrigena	
	Granulicella mallensis	
	Ilumatobacter fluminis	
	Krasilnikovia cinnamomea	
	Mycobacterium celatum	
	Mycobacterium conspicuum	
	Mycobacterium shimoidei	
	Nocardia alba	
	Oryzihumus leptocrescens	
	Phenylobacterium falsum	
BA-CR-RH	*Actinomadura miaoliensis*	*Duganella phyllosphaerae*
	Altererythrobacter namhicola	*Kaistia defluvii*
	Devosia neptuniae	*Lysobacter enzymogenes*
	Devosia riboflavina	*Luteibacter rhizovicinus*
	Flavitalea gansuensis	*Microbacterium xylanilyticum*
	Flavobacterium succinicans	*Mucilaginibacter composti*
	Lysobacter oligotrophicus	*Mucilaginibacter polysacchareus*
	Nitratireductor basaltis	*Rhizobium pusense*
	Promicromonospora umidemergens	*Rhodanobacter umsongensis*
	Sphingobacterium kitahiroshimense	*Streptomyces neyagawaensis*
	Sphingopyxis bauzanensis	
	Steroidobacter agariperforans	
	Streptomyces ardus	
	Streptomyces yanglinensis	
	Thermomonas brevis	
BW-CR-BU	*Simiduia areninigrae*	*Streptomyces stelliscabiei*
	Flavobactreium ginsenosidimutans	*Streptomyces rochei*
	Streptomyces durmitorensis	*Streptomyces glomeratus*

	Chryseobacterium haifense	
BW-CR-RH	*Sphingobacterium multivorum*	*Streptomyces scabiei*
	Brevicaterium antiquum	*Streptomyces humidus*
	Pedobacter arcticus	*Streptomyces aburaviensis*
BA-H-BU	*Bacillus benzoevorans*	NA
	Dyella marensis	NA
	Kribbella swartbergensis	NA
	Leifsonia shinshuensis	NA
	Streptomyces vitaminophilus	NA
BA-H-RH	*Clostridium butyricum*	NA
	Inquilinus limosus	NA
	Sphingomonas changbaiensis	NA
	Sphingomonas polyaromaticivorans	NA
	Sphingobacterium cladoniae	NA
BW-H-BU	*Candidatus Nitrososphaera SCA1145*	NA
	Gaiella occulta	NA
	Mesorhizobium camelthorni	NA
BW-H-RH	*Agromyces atrinae*	NA
	Amycolatopsis xylanica	NA
	Angustibacter luteus	NA
	Dyella japonica	NA
	Flavobacterium araucananum	NA
	Flavobacterium cheonanense	NA
	Methyloversatilis universalis	NA
	Mycobacterium fluoranthenivorans	NA
	Ramlibacter henchirensis	NA
	Rhodococcus kroppenstedtii	NA
	Rhodococcus tukisamuensis	NA
	Sphingopyxis witflariensis	NA
	Streptomyces malaysiensis	NA
	Saccharopolyspora shandongensis	NA

NA, Not applicable as No study was conducted at the Harrington location in 2015. BA = barley, BW = buckwheat, CR = Cross Road, H = Harrington, BU = bulk, RH = rhizosphere.

Table 8.2. Common bacterial species found in all samples during the 2014 and 2015 growing seasons.

Common bacterial species	
Achromobacter spanius	*Nocardioides tritolerans*
Acidipila rosea	*Novosphingobium subterraneum*
Acinetobacter calcoaceticus	*Novosphingobium tardaugens*
Acrocarpospora corrugata	*Novosphingobium lindaniclasticum*
Actinomadura glauciflava	*Oligoflexus tunisiensis*
Actinomadura miaoliensis	*Oryzihumus leptocrescens*
Actinomycetospora chiangmaiensis	*Methylotenera mobilis*
Actinoplanes globisporus	*Microvirga aerophila*
Actinospica acidiphila	*Modestobacter multiseptatus*
Actinospica robiniae	*Mucilaginibacter dorajii*
Advenella incenata	*Mucilaginibacter gossypii*
Aeromicrobium ginsengisoli	*Mucilaginibacter gracilis*
Agromyces atrinae	*Mucilaginibacter sabulilitoris*
Alcanivorax pacificus	*Mycobacterium fluoranthenivorans*
Altererythrobacter atlanticus	*Mycobacterium frederiksbergense*
Altererythrobacter namhicola	*Mycobacterium hodleri*
Altererythrobacter troitsensis	*Mycobacterium insubricum*
Amycolatopsis australiensis	*Mycobacterium llatzerense*
Amycolatopsis pigmentata	*Mycobacterium vaccae*
Arthrobacter niigatensis	*Mycoplana ramosa*
Asticcacaulis biprosthecium	*Paenibacillus daejeonensis*
Bacillus longiquaesitum	*Paenibacillus pectinilyticus*
Blastococcus aggregatus	*Panacagrimonas perspica*
Blastococcus endophyticus	*Pantoea agglomerans*
Blastococcus saxobsidens	*Pantoea brenneri*
Bosea genosp.	*Paracoccus alcaliphilus*
Bradyrhizobium canariense	*Pedobacter borealis*
Bradyrhizobium elkanii	*Pedobacter koreensis*
Bradyrhizobium iriomotense	*Pedobacter panaciterrae*
Brevundimonas bullata	*Pedomicrobium manganicum*
Bryocella elongata	*Peredibacter starrii*
Burkholderia caledonica	*Phenylobacterium immobile*
Burkholderia terrestris	*Povalibacter uvarum*
Catenulispora yoronensis	*Promicromonospora umidemergens*
Caulobacter henricii	*Pseudolabrys taiwanensis*
Cellvibrio gandavensis	*Pseudonocardia ammonioxydans*

Chitinophaga ginsengisegetis
Chitinophaga niabensis
Curvibacter fontanus
Deinococcus yunweiensis
Devosia insulae
Devosia neptuniae
Dokdonella soli
Duganella zoogloeoides
Dyella thiooxydans
Edaphobacter modestum
Edaphobacter modestus
Enhydrobacter aerosaccus
Filomicrobium fusiforme
Flavitalea gansuensis
Flavobacterium anatoliense
Flavobacterium araucananum
Flavobacterium cheonanense
Flavobacterium glaciei
Flavobacterium hercynium
Flavobacterium nitratireducens
Flavobacterium pectinovorum
Glycomyces arizonensis
Granulicella aggregans
Granulicella pectinivorans
Herbaspirillum psychrotolerans
Hyalangium minutum
Hyphomicrobium vulgare
Illumatobacter nonamiense
Kribbella sancticallisti
Labrys wisconsinensis
Lapillicoccus jejuensis
Lentzea jiangxiensis
Luteibacter rhizovicinus
Luteimonas vadosa
Lysobacter dokdonensis
Lysobacter enzymogenes
Lysobacter oligotrophicus
Lysobacter pocheonensis
Marmoricola korecus
Marmoricola scoriae
Massilia aerilata
Pseudonocardia spinosispora
Pseudonocardia tropica
Pseudonocardia xinjiangensis
Pseudoxanthomonas yeongjuensis
Pullulanibacillus naganoensis
Rhizobium radiobacter
Rhodanobacter lindaniclasticus
Rhodanobacter spathiphylli
Rhodanobacter terrae
Rhodanobacter umsongensis
Rhodococcus fascians
Roseomonas aquatica
Roseomonas riguiloci
Rubrivivax gelatinosus
Rugamonas rubra
Saccharopolyspora tripterygii
Sphingobacterium cladoniae
Sphingobacterium shayense
Sphingobium boeckii
Sphingomonas asaccharolytica
Sphingomonas faeni
Sphingomonas jaspsi
Sphingomonas oligophenolica
Sphingomonas sediminicola
Sphingomonas wittichii
Sphingopyxis alaskensis
Sphingoterrabacterium pocheensis
Sporichthya polymorpha
Stackebrandtia albiflava
Stenotrophomonas rhizophila
Steroidobacter denitrificans
Streptacidiphilus carbonis
Streptomyces abietis
Streptomyces albiaxialis
Streptomyces ardus
Streptomyces armeniacus
Streptomyces cyaneus
Streptomyces eurythermus
Streptomyces ferralitis
Streptomyces griseoplanus
Streptomyces nanshensis

Methylibium petroleiphilum	*Streptomyces olivochromogenes*
Methylophilus flavus	*Streptomyces paucisporeus*
Nakamurella flavida	*Streptomyces phaeoluteigriseus*
Nakamurella multipartita	*Streptomyces rishiriensis*
Nitratireductor basaltis	*Streptomyces sannurensis*
Nitrosospira multiformis	*Streptomyces yanglinensis*
Nocardioides daphniae	*Streptomyces cacaoi*
Nocardioides halotolerans	*Terrabacter carboxydivorans*
Nocardioides hwasunensis	*Terriglobus roseus*
Nocardioides islandensis	*Thermomonas brevis*
Nocardioides marinquilinus	*Variovorax paradoxus*
Nocardioides mesophilus	*Woodsholea maritima*

In addition, 3 species were found only in the buckwheat rhizosphere soil at both the Harrington and Cross Road locations, and referred to as buckwheat rhizosphere bacteria. These bacteria were identified as *Methylophilus flavus, Saccharopolyspora tripterygii*, and *Deinococcus yunweiensis* and belong to the *Proteobacteria, Actinobacteria*, and *Deinoccocus-Thermus* phyla, respectively (Table 9).

Table 9. The unique species found only in the buckwheat rhizosphere soil at both the Harrington and Cross Road locations.

Phylum	Class	Order	Family	Genus	Species
Proteobacteria	*Betaproteobacteria*	*Methylophilales*	*Methylophilaceae*	*Methylophilus*	*Flavus*
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Pseudonocardiaceae*	*Saccharopolyspora*	*Tripterygii*
Deinococcus-Thermus	*Deinococci*	*Deinococcales*	*Deinococcaceae*	*Deinococcus*	*yunweiensis*

Taken together, a total of 5 species belonging to *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were only observed in both barley and buckwheat rhizosphere soils, and were thus considered as species unique to rhizosphere soils (Table 10). In addition, 2 species were found only in the buckwheat bulk soils from Harrington and Cross Road in 2014 (Table 11), whereas 2 species were observed in the barley and buckwheat bulk soils during the 2014 growing season, and referred to as unique to bulk soils (Table 12).

Table 10. Species found only in the barley and buckwheat rhizosphere soils.

Phylum	Class	Order	Family	Genus	Species
Actinbacteria	*Actinobacteria*	*Actinomycetales*	*Pseudonocardiaceae*	*Pseudonocardia*	*Tropica*
Protobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	*asaccharolytica*
Protobacteria	*Oligoflexia*	*Oligoflexales*	*Oligoflexaceae*	*Oligoflexus*	*Tunisiensis*
Protobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	*Bosea*	*genosp.*
Bacteroidetes	*Sphingobacteriia*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Sphingoterrabacterium*	*Pocheensis*

Table 11. Species found only in buckwheat bulk soils at the Harrington and Cross Road locations in 2014.

Phylum	Class	Order	Family	Genus	Species
Protobacteria	*Gammaprotobacteria*	*Oceanospirillales*	*Alcanivoraceae*	*Alcanivorax*	*Pacificus*
Actinobacteria	*Actinobacteria*	*Glycomycetales*	*Glycomycetaceae*	*Stackebrandtia*	*Albiflava*

Table 12. Species found to be common to barley and buckwheat bulk soils in 2014.

Phylum	Class	Order	Family	Genus	Species
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Methylobacteriaceae*	*Microvirga*	*Aerophila*
Firmicutes	*Bacilli*	*Bacillales*	*Paenibacillaceae*	*Paenibacillus*	*Daejeonensis*

2.4 Discussion and Conclusion

Microbial communities in the soil play an essential role in plant health, soil fertility and nutrient cycling (Yang et al., 2017; Mends et al., 2014; García-Salamanca et al., 2012). It has been reported that microbial diversity can be affected by different factors including plant genotype, soil type, and growing phenology (Yang et al., 2017). In this context, the rhizosphere is the main contact zone between the soil and soil microbiomes, and is defined as the narrow zone of soil that is influenced by plant roots (Yang et al., 2017; García-Salamanca et al., 2012; Dennis, Miller & Hirsch, 2010; Jones et al., 2009; Trujillo et al., 2015). It is now established that several factors, including plant species that can affect the microbial diversity either directly (root exudation) or indirectly by affecting the soil environmental conditions (moisture, pH, and soil nutrients), which in turn influence the microbial populations (Maul and Drinkwater, 2010; Linkun et al., 2015). In this study, 16S metagenomics was performed using an Ion Torrent PGM platform to assess and identify the microbial diversity in the bulk and rhizosphere soils in two crops (buckwheat, barley) at two locations (Harrington and Cross road-Stratford) over two years. The study identified a total of 27 phyla in the bulk and rhizosphere soils of the two locations investigated and *Protobacteria* was found to be the most dominant phylum. Moreover, variations in the microbial composition and abundance were observed between bulk and rhizosphere soils in each crop at each location.

An estimation of the required sequencing depth needed to characterize a particular microbiome is important to achieve for any given study (Zaheer et al., 2018). In the current study, diagnostics of sequencing accuracy and depth were performed, and consistent numbers of reads

were obtained independent of loading chip types used, albeit slight variations were observed between individual runs mainly due to technical errors such as chip loading. In addition, the number of libraries sequenced in each run varied (4 - 7 libraries). Furthermore, rarefaction curves used to estimate the species richness as a function of sequencing depth reached their plateau in each sample at 299 sequences/sample, suggesting a sufficient sequencing depth was achieved in this study as also reported by (Zaheer et al., 2018), providing a good representation of comparative microbial diversity among samples. Nonetheless, the short 16S RNA amplicons evaluated in this study may have not been enough to provide the full coverage of the microbiome spectrum in the samples investigated.

The microbial diversity is mainly described by alpha diversity and beta diversity (Goodrich et al., 2014). Alpha diversity measures the diversity associated with a single sample for example, number of OTUs, Shannon Index, and rarefaction curve, etc. (Mayo et al., 2014) and is essential to compare the total diversity in different communities (Lozupone and Knight, 2008; Kuczynski et al., 2011). In this study, the microbiome diversity estimated in terms of alpha diversity in buckwheat was found to be higher in the rhizosphere soil samples compared to the bulk soil, independent of the growing year, and a good correlation was also observed between the Choa1, observed species, Shannon and Simpson estimators for the alpha diversity in barley. These observations indicate that buckwheat rhizosphere may attract some soil microbiome through its rhizo-deposits. Similar observations have been reported in the study conducted by Garcia-Salamanca et al. (2012) showing that the microbial diversity in the maize rhizosphere soil was higher than in the bulk soil and that plants can alter the microbial composition in the soil (Garcia-Salamanca et al., 2012). Beta diversity provides a measure of similarity or dissimilarity between sample taxonomic diversity by characterizing the number of species present in different samples (Morgan and Huttenhower, 2012; Goodrich et al., 2014; Kuczynski et al., 2010; Lozupone and Knight, 2008). The PCoA plot showed that most of the samples are similar to one another, except for single replicates in three samples (BACR 2014 RH2, BACR2014 BU3, and BWH RH2) which were far from others, probably as a result of technical bias (slightly higher number of reads in these runs) compared to their other counterpart replicates. In the best scenario, one would expect that the replicates from each sample to cluster together and separate from other groups of samples. This was not clearly indicated by the PCoA analysis shown in the current study. This finding reflects the low microbial diversity observed between crops (barley and

buckwheat) and soil types (bulk and rhizosphere) at most taxonomic levels (phyla, order, family) since most samples were clustered without large spatial variations as shown in the PCoA, thus masking the albeit statistically significant variation observed between barley and buckwheat in terms of community structure in the bulk and rhizosphere soils of barley or buckwheat at the species levels in 2014 and 2015.

A total of 27 phyla were identified in the current study, and members of the *Proetobacteira* were the most abundant, followed by *Actinobacteria*. *Proteobacteria*, along with *Actinobacteria, Acidobacteria Bacteroidetes, Firmicutes, Deinococcus-Thermus* and *Crenarchaeota* were the 7 phyla in which species were identified confidently. *Proteobacteria* are mostly Gram-negative and many are accountable for nitrogen fixation and the production of polycyclic aromatic hydrocarbons (Yang et al., 2017; García-Salamanca et al., 2012). *Proteobacteria* are morphologically, physiologically and metabolically diverse and can play an important role in soil C, N, and S cycling (Gardner et al., 2011). Even though *Proteobacteria* were found in both in bulk and rhizosphere soils, they were more common in the rhizosphere. Similar findings have been previously reported, stating that *Proteobacteria* are the most dominant phylum in the rhizosphere, perhaps because of their rapid growth rates (Sharma et al., 2005; Yang et al., 2017; Johnston-Monje et al., 2016). *Actinobacteria* are widely represented in the environment (water and soil) and play an essential role in organic matter decomposition and formation of humus (Yang et al., 2017; Buee et al., 2009). In this study, phylum *Actinobacteria* was the second most abundant in the bulk and rhizosphere soils of the two crops (buckwheat and barley). Specifically, in the buckwheat rhizosphere *Actinobacteria* accounted for 55 % of the mean density. Trujillo and colleagues reported that *Firmicutes* and *Actinobacteria*, Gram-positive bacteria such as *Bacillus, Micromonospora, Streptomyces* and others, are beneficial for agriculture specifically as plant growth-promoting bacteria, in bioremediation activities and are also considered as excellent biocontrol microbes (Trujillo et al., 2015; Sharma et al., 2005). Also, a study conducted by Sharma and his collaborators investigated the bacterial community in the rhizosphere soil of legumes and demonstrated that the bacterial communities present in the rhizosphere of legumes were effective biocontrol agents against legume pathogens that affect plant growth and development (Sharma et al., 2005). The current study shows that the major phyla found in the soils are *Proteobacteria, Actinobacteria, Bacteroidetes* and *Firmicutes,* in agreement with previous studies (Yang et al., 2017; Vurukonda and Stefani, 2018). According to Trujillo et al. (2015), among Gram-positive

bacteria *Firmicutes* and *Actinobacteria* including *Bacillus*, *Micromonospora* and *Streptomyces* have a positive influence on plant growth promotion, bioremediation activities, as well as biocontrol (Trujillo et al., 2015). One of the unique species found only in barley bulk soil at the Harrington location is *Streptomyces vitaminophilus*, a species reported to produce halogenated antibiotics named pyrrolomycins (Mahan et al., 2016). However, more studies are needed to confirm whether this species is specific to barley. Moreover, *Streptomyces* species have been reported to greatly influence the microbial population structure due to their ability to produce natural substances playing an important role as biocontrol agents by killing or suppressing plant pathogens (Vurukonda et al., 2018; Schmidt and Spiteller, 2017). According to Schmidt and Spiteller (2017), *Streptomyces aburaviensis* produces antibiotics. In the current study, *Streptomyces aburaviensis* was found to be unique to buckwheat rhizosphere soils at Cross Road. However, more studies are still required to confirm this finding for a potential applications in the biomedical area. In addition, *Streptomyces cacaoi* was found in the buckwheat rhizosphere at the Cross Road location in 2014 and 2015. This species was reported in a previous study as antagonistic bacteria that play an important role as biological control agents against plant nematodes (Yoon et al., 2012). Nonetheless, some *Streptomyces* species are known as plant pathogens, causing serious plant diseases (Vurukonda et al., 2018; Komeil et al., 2014; Leiminger et al., 2013). *Streptomyces scabiei* or *Streptomyces scabies* is a clear example and was found only in buckwheat rhizosphere soils at Cross Road. This species is known as plant pathogenic bacterium causing common scab that decreases potato production and causes economic losses in many potato fields by producing toxins called thaxtomins (Komeil et al., 2014; Leiminger et al., 2013). This finding, if confirmed, is intriguing in the context of high scab pressure in potato growing areas where buckwheat is promoted as rotation crop for wireworm control. Indeed, a previous study by Wiggins and Kindel (2005) showed a high scab disease incidence in potato after two years of green manure treatment as performed in the current study. Pending questions that need to be answered are: Is buckwheat a host for scab or does its root exudates attract scab. On the other hand, a one-year green manure treatment using buckwheat and canola resulted in a reduction of common scab, increased potato yield, and an increase in the density and activity of *Streptomyces* toward different soil borne pathogens, weeds and nematodes (Larkin and Honeycutt, 2012; Laznik et al., 2014).

Paenibacillus species play essential roles in sustainable agriculture and biotechnology (Grady et al., 2016). Many *Paenibacillus* species are able to promote crop growth by biological nitrogen fixation, producing antimicrobial compounds that control plant pathogens and insects including bacteria, fungi, nematodes, and viruses (Grady et al., 2016). Moreover, *Paenibacillus* can trigger induced systemic resistance (ISR) which is a latent defense mechanism occurring in plant tissues providing enhanced protection against a variety of pathogens and pests. According to Kobayashi et al. (2015) *Paenibacillus*, *Bacillus, Rhodococcus, Arthrobacter,* and *Streptomyces* have the ability to control common scab (Kobayashi et al., 2015). Our study also identified *Paenibacillus, Rhodococcus* and *Arthrobacter* in one or both crop root system soils, but their specific roles in scab control or soil health remains to be clarified.

In the current study, 3 OTUs at the species level were found to be unique to the buckwheat rhizosphere soil at both the Harrington and Cross Road (Stratford) locations. These species were *Methylophilus flavus, Saccharopolyspora tripterygii* and *Deinococcus yunweiensis* and were characterized as non-pathogenic bacteria (endophytic or symbiotic) (Gogleva et al., 2010; Li et al., 2009; Zhang et al., 2007). As reported by Gogleva et al. (2010), *Methylophilus flavus* is known as a methylotrophic bacterium, and has been reported to have beneficial effects on host plants (entophytic) and was suggested for sustainable agriculture (Kumar et al., 2016; Meena et al., 2012) because it produces biocontrol agents that reduce plant diseases and predation by insects (Melnick et al., 2013). A study conducted by Li and colleagues demonstrated that *Saccharopolyspora tripterygii* is an endophytic actinomycete (Li et al., 2009). These microorganisms play an essential role in supporting plant growth under different conditions (drought, heat, nutrient-poor conditions) and in disease resistance either directly or indirectly (Santoyo et al., 2016; Udwary et al., 2011;Trujillo et al., 2015; Kandel et al., 2017). Zang and colleagues reported that *Deinococcus yunweiensis,* a Gram-negative stain, is one of the species belonging to genus *Deinococcus* and is highly resistant to environmental hazards (Zhang et al., 2007). This species was found in buckwheat rhizosphere soil. Previous studies indicate that most endophytes interacting with plants originate from the rhizosphere or phyllosphere (Kumar et al., 2016; Trujillo et al., 2015; Dudeja et al., 2012). According to Santoyo et al. (2016), the endophytic bacterial community is considered as a rhizosphere bacterial community or root-associated bacterial population (Kumar et al., 2016; Santoyo et al., 2016; Marquez-Santacruz et al., 2010).

Soil microbial diversity is strongly influenced by plant species and microbial characteristics have been reported to change from season to season (Siles and Margesin, 2017). Therefore, crop rotation is considered as an important factor for shaping the microbial diversity in the soil, either bulk or rhizosphere soils. In the current study, a more diverse soil microbiome was observed in barley compared to buckwheat, which is consistent with previous findings (Larkin and Honeycutt, 2005). However, beneficial microbial species were found in both crops (barley and buckwheat), (Trujillo et al., 2015; Sharma et al. ,2005). Additionally, this study showed that buckwheat contributed to a reduction in taxonomic richness by 20% and 32% at the family and genus levels, respectively, over two growing seasons compared to barley (13% and 18% at the same taxonomic levels). This finding supports the beneficial role for buckwheat as a rotation crop in terms of reduction of bacterial loading. However, how this reduction is achieved requires further study.

In conclusion, this 2-year study has documented changes in soil microbial communities associated with specific rotation crops. These findings are in general agreement with previous studies but further demonstrate the positive effects of buckwheat in reducing bacterial loads in the field. Some bacterial species that may have a biomedical application were also detected, thus setting the way for a potential use of crops to isolate bacteria producing high value antibiotics. Taken together, we demonstrated that the root system of buckwheat influences the structure of the microbiome in the rhizosphere as hypothesized. However, more studies are required to assess the mechanisms by what this is achieved.

CHAPTER THREE: Correlation between buckwheat rhizosphere microbiome structure and wireworm density

3.1 Introduction

The rhizosphere is the soil closely attached to the roots. Known as a complex environment where plants and millions of microbes interact with each other (Sugiyama et al., 2014; Yang et al., 2017; García-Salamanca et al., 2012; Dennis, Miller and Hirsch, 2010; Jones et al., 2009; Haldar and Sengupta, 2015). The soil microbiomes containing bacteria and fungi constitutes a very small portion of the composition of the soil but it plays a significant role in the cycling of nutrients such as N, P, and S, and other ecosystem functions (Yang et al., 2016; Jacoby et al., 2017). The microbes in the rhizosphere have been shown to have intense and important effects on plant growth and health (Bargaz et al., 2018; Jacoby et al., 2017). For instance, mycorrhizae and rhizobia provide P and N, respectively, and microbes called plant-growth-promoting rhizobacteria (PGPR) exert both direct as well as indirect effects on plant growth including the prevention of colonization by pathogens and the modulation of plant immunity (Bargaz et al., 2018; Sugiyama et al., 2014; Jacoby et al., 2017). These rhizosphere microbes are considered as prominent components for agricultural sustainability, contributing to decrease the use of fertilizers and pesticides. Plants have the ability to accommodate microorganisms in the rhizosphere by providing a platform and nutrients, mainly root exudates which account for up to 40% of photosynthates. In addition to the microclimate and chemical properties of the soils, resident plants exert influences on rhizosphere microbial communities. Microbial communities have been found to be dependent on the plant species grown in the same type of soil, indicating a close interaction between plants and rhizosphere microbial diversity (Sugiyama et al., 2014). These microbiomes contribute to soil structure stabilization, organic residue accumulation, nitrogen fixation, and removal of toxins. In addition, the populations of microbial species in the rhizosphere soil significantly contribute to the maintenance of crop health, and they are considered as one of the greatest biological indicators of soil quality changes (Jacoby et al., 2017). Recently, studies have paid close attention to soil microbial diversity as well as its impact on agricultural ecosystems (Umesha et al., 2017; Yang et al., 2016; Bargaz et al., 2018; Jacoby et al., 2017).

Soil microbial communities interact with the rhizosphere of plants, and many factors have been reported to affect microbial diversity in that specific space (Qiao et al., 2017). Qiao and his colleagues reported that plant growth, development and plant immune system are influenced by the microbial diversity in the rhizosphere via different mechanisms (Qiao et al., 2017). These rhizosphere microorganisms are considered as prominent components to maintain agricultural sustainability by decreasing the use of pesticides (Sugiyama et al., 2014). Previous studies report that cropping systems can impact soil microbial communities, and that the abundance and community structure of soil bacterial taxa changed in response to changes in management practices (Yang et al., 2016; Niu et al., 2017).

Microbial diversity in the soil may also be influenced by different agricultural practices such as sustainable management changes in land use (Hol et al., 2015; Mendes et al., 2014; Yang et al., 2016; Trivedi et al., 2016), fertilization, and row ratio of the intercrops (Umesha et al., 2017). Recently, several factors including: soil type, nutrition, management practice, soil properties, varietal differences within a species, plant age, plant species, as well as plant genotype have been reported to affect bacterial diversity in the rhizosphere (Mendes et al., 2014; Yang et al., 2016; Mahoney et al., 2107). The study conducted by Yang et al. (2016), demonstrated that cropping systems and crop species had significant effects on soil microbial diversity in the rhizosphere. For example, *Empedobacter brevis* was found to be present in the carrot rhizosphere (Yang et al., 2016). Furthermore, a strong correlation was found between aboveground plant diversity and underground microbial diversity (Yang et al., 2016). Several studies have been conducted on the microbial diversity of the rhizosphere in many crops such as peanut, wheat, maize, soybean, cucumber, onion, cotton and garlic and variations in microbial diversity in the rhizosphere were found to be dependent on the crops used and cultivation systems (Yang et al., 2016; Qiao et al., 2017). The microbial diversity and its composition (relative abundance of major bacterial phyla) have been reported to be affected in response to agricultural management systems (Trivedi et al., 2016). One-year green manure treatment of buckwheat and canola have led to a reduction of common scab to increase potato yield, to increase the density and activity of *Streptomyces* against different soil borne pathogens, weeds and nematodes (Larkin and Honeycutt, 2012; Laznik et al., 2014). As described by Sugiyama et al. (2014), additional studies on metabolic activities of soybean (root exudation) as well as the physiological functions of these rhizobacteria on plant growth will likely clarify the mutual interactions between plants and rhizo-

sphere microbiomes in the field for better utilization of rhizosphere bacteria for sustainable agriculture (Sugiyama et al., 2014).

While understanding microbial diversity is important, its effect on wireworm needs to be understood. Wireworms are soil dwelling larvae of click beetles (Coleoptera: Elateridae) (Furlan, 2005; Barsics et al., 2013; Laznik et al., 2014; Knodel & Shrestha, 2018). They live in the soil and feed on different parts of the plant (roots, root nod-ules, stems, leaves, flowers, pods, and seeds) (Knodel & Shrestha, 2018). Wireworms cause damage to many plants including carrots, sugar beet (Laznik et al., 2014), potatoes, and maize, affect the crop value and reduce yields (Ansari et al., 2009; Vernon et al., 2013; Traugott et al., 2014; Barsics et al., 2013; Keiser et al., 2012; Parker and Howard, 2001; Ritter and Richter, 2013; van Herk and Vernon, 2014; Vernon and van Herk, 2013). Previouse studies reported that control of wireworms is chalenging, mainly because of its long life cycle (2-6 years) (Figure A3) (Studacher et al., 2013; Blackshaw and Kerry, 2008; Saussure et al., 2015; Blackshaw and Hicks, 2013; Blackshaw and Vernon, 2006). Different chemical pesticides are commonly used to address the wireworm issues and control the pest. These pesticides include neonicotinoids, pyrethroids, as well as a phenyl pyrazole. However, chemical pesticides can badly affect human health and the environment, and their uses have been restricted in many countries (Saussure et al., 2015; Geiger et al., 2010; Traugott et al., 2015). Therefore, the use of environmentally friendly plant protection methods (Saussure et al., 2015; Staudacher et al., 2013; Laz-nik et al., 2014) such as crop rotations have been suggested (Alyokhin et al., 2013). According to a recent study using brown mustard or buckwheat as rotation crops in potato fields in Prince Edward Island, a decrease in wireworm populations was observed (Alyokhin et al., 2013; Noronha, 2011). As indicated by Ortiz-Castro et al. (2009), interactions exist between plants, the soil microflora and microbiome, and understanding of these plant-microbiome interactions can lead to improved and healthy agricultural production systems (Ortiz-Castro et al., 2009).

In the previous chapter, it was reported that the rhizosphere of buckwheat affected the composition of the soil microbiome differently compared to barley as rotation crop. However, it is not known whether this difference has an impact on wireworm populations in the soil. The objective of this chapter is to determine if there is a correlation between the structure of the microbiome of the rhizosphere and wireworm density.

3.2 Materials and methods

3.2.1 Study site and characterization

The locations used for this study consisted of two fields: 1) Cross Road, characterized by a high wireworm infestation and located at Stratford in Prince Edward Island and 2) Harrington, characterized by a low wireworm infestation and located at the Harrington Research farm in Prince Edward Island. Three soil samples collected at the 0 –15 cm depth at each location and send to PEI analytical lab for pH determination. The soil pH for each sample was determined using a 1:1 ratio of soil to water (10g soil: 10 ml water).

3.2.2 Determination of wireworm popbulation

To determine the density of wireworm, a pilot wireworm density assessment was performed. Wireworm baits were placed in the fields for seven days. Baits consisted of a hole (3.5 inches across and 5.5 inches deep) in which a wired flag was placed in the center along with ¾ cup of cut carrots as bait, covered and packed with soil. (Figure 15A-E). After seven days, the bait was recovered using a probe at the location of the flag. The carrot samples in the bait were collected and placed in bags (Figure 16 A-D). These bags were labeled with appropriate tags, tied and stored. Then, the wireworms were collected from the baits and counted from each bag/sample from each location and crop (Table 13).

Table13. Number of baits placed in each location and plot per year.

Year	Location	Plot	Number of Baits
2014	Cross Road	Barley	43
	Cross Road	Buckwheat	41
	Harrington	Barley	12
	Harrington	Buckwheat	20
2015	Cross Road	Barley	29
	Cross Road	Buckwheat	36
	Harrington	Barley	10
	Harrington	Buckwheat	10

Figure 15. Preparation of wireworm baits in the field. A) Installation of the bait probe; B) Insertion of wire flag in the center of the hole as a site marker; C) Addition of ¾ cup of cut carrots in the hole around the flag; D) Covering the bait with soil; E) View of the bait location the field.

Figure 16. Wireworms bait collection in the field. A) Collection of the baits using a probe to locate the marker flag; B) Recovery of the bait from the ground using the probe; C) Placing the collected baits into a sample bag ; D) Labeling of the sample bag.

3.3 Results

The soil pH at each location in all field locations (Cross Road and Harrington sites) was found to be slightly acidic. The soil pH at the Cross Road site in 2014 growing season was 5.69 for the buckwheat plot and 5.88 for the barley plot. At the Harrington site, the soil pH values of the plots (buckwheat and barley) were 5.8 in 2014 and 6.5 in 2015.

After seven days of baiting in each field at both the Harrington and Cross Road (Stratford) locations during each of the two growing seasons (2014 and 2015), a lower number of wire-worms were found in each crop at the Harrington location compared to Cross Road (wireworm infested soil) as anticipated (Table 14). A substantial decrease in wireworm counts was observed in the barley field from 2014 to 2015 at both the Cross Road and Harrington locations, with 40 – 60 % reduction, respectively. In the buckwheat field, a 75% reduction in the wireworm counts was observed at the Harrington location but only a reduction of 7% at the Cross Road location (Table 14) (Figure A4, Appendix 3).

Table 14. Observed wireworm number per field at different locations in barley and buckwheat plots during the 2014 and 2015 growing seasons.

Location	**Barley**		**Buckwheat**	
	2014	**2015**	**2014**	**2015**
Cross Road	**47**	**28**	**28**	**26**
Harrington	**5**	**2**	**4**	**1**

3.4 Discussion and conclusion

Wireworm has become an issue in all potato growing areas in Canada (van Herk and Vernon, 2014; Vernon et al., 2009) and its management is not straightforward especially with limited pesticide treatment options (Vernon et al., 2009). In the current study, a pilot wireworm trapping survey was performed to assess the presence of wireworms following buckwheat and barley as rotation crops. In this study, a reduction in the number of wireworms was observed in barley and buckwheat fields.

The rhizosphere is defined as the portion of the soil that is influenced directly by plant roots and is considered as an enriched region by microorganisms (Ahkami et al., 2017). According to Valverde and Ramirez (2014), the microbial diversity of the rhizosphere has beneficial effects on plants. For example, plant growth promoting bacteria are used as agronomic inputs to increase crop yield (Valverde and Ramirez, 2014). In the barley field, surveyed in our study, a reduction in the number of wireworms was observed whereas only a modest reduction was noted in the buckwheat field, which was unexpected. In fact, as a pilot experiment the design and number of samples used were not appropriate enough to draw a solid conclusion. Nonetheless, in this study, buckwheat had a modest effect on wireworm counts per field Moreover, non-pathogenic (entophytic) bacterial species were found to be associated with the rhizosphere of buckwheat. These species have been reported to enhance plant growth, protect plant from pathogens, and reduce plant pests including diseases and insects (Kumar et al., 2016; Melnick et al., 2013; Kandel et al., 2017). However, whether these species directly affect wireworms is still to be determined.

As far as barley is concerned, a reduction was observed in the number of wireworms that were captured when compared with buckwheat. As reported in different studies (Carter et al., 2009; Larkin et al., 2012), crop rotations play an essential role to improve plant growth by suppressing soil-borne diseases and pests. Appropriate rotation crops can positively influence soil microbial diversity (Carter et al., 2009; Larkin et al., 2012). The current study tends to show such as a trend. Indeed, it is known that rotation reduces disease incidence through the disruption of host and non-host interactions (Latz et al., 2015; Wang et al., 2017). In the current study however, the same crops (barley and buckwheat) were planted in the same field for two consecutive years, precluding the observation of the host/non-host phenomenon. It is known that each plant

species displays its particular microbial community (Costal et al., 2004). Latz et al. (2015) reported that some plants increase root exudation and alter the composition of their exudate which in turn affects rhizosphere microbial communities. Moreover, other studies report that crop rotation can help to reduce wireworm populations and damage in different crops, and induced changes in the biological properties of soil (Kandel and Shrestha, 2018; Traugott et al., 2015; Carter et al., 2009).

In conclusion, after two years of cultivation of barley and buckwheat in the same fields, a modest reduction in wireworm density was observed as hypothesized for this study. However, the direct link associating this wireworm density reduction to the observed microbial diversity and how this reduction is operated in terms of mechanisms in each crop remain to be elucidated. It will be of interest to carry out a more robust wireworm density study using buckwheat as cover crop and determine the factors that affect wireworm density.

CHAPTER FOUR : General discussion and conclusions

4.1 General Discussion

Crop production plays an essential role across the world to meet global food requirements (Knodel and Shrestha, 2018). However, crop productions have been influenced by different factors including climate change, abiotic and biotic stresses (Knodel and Shrestha, 2018; Ahkami et al., 2017; Saussure et al., 2015). In Canada, wireworm has become a major concern in potato production areas (Vernon et al., 2013) and limited control agents including chemical pesticides (Saussure et al., 2015; Larkin and Griffin, 2007) have increased the need for alternative strategies such as crop rotation (Saussure et al., 2015; Larkin et al., 2012). In this study, a two year rotation was used to determine the microbial diversity in buckwheat rhizosphere soils compared with barley and brown mustard, and to determine if there is a correlation between the rhizosphere microbiome structure and wireworm density. Because brown mustard was only grown at Harrington location for one growing season, and no comparative data was available for further analysis. Therefore, brown mustard was not included in the results. Current molecular strategies enable researchers to characterize microbial populations from complex environments such as soils and marine ecosystems (Sharma et al. ,2005; Knief, 2014). Among these strategies, 16S metagenomic sequencing was performed in the current study to describe the microbial diversity in bulk and rhizosphere soils, and the technique is of great potential for analyzing the structure and diversity of the soil microbial community. The study reported that the 2-year crop rotation led to changes in the soil microbial communities associated with specific rotation crops and a corresponding reduction in wireworm density was also observed. Thus, our study showed that buckwheat root system may have an influence on the structure of the microbiome in the rhizosphere as hypothesized.

In this study, a total of 27 phyla were identified, and *Proetobacteria* was the most abundant, followed by *Actinobacteria*. The current system using metagenomics approach allowed us to identify species in seven phyla including *Proetobacteria, Actinobacteria, Acidobacteria, Bacteroidetes, Firmicutes, Deinococcus-Thermus* and *Crenarchaeota,* in agreement with previous studies in terms of diversity (Yang et al., 2017; Vurukonda and Stefani, 2018). Even though representatives of *Proteobacteria* were found in both bulk and rhizosphere soils, they were more

abundant in the rhizosphere compared with the bulk soils. Similar findings have been previously reported, suggesting that *Proteobacteria* are the most dominant phylum in the rhizosphere, perhaps because of their rapid growth rates (Sharma et al., 2005; Yang et al., 2017; Johnston-Monje et al., 2016). *Actinobacteria* are widely present in the environment and play an essential role in decomposition and formation of humus (Yang et al., 2017; Buee et al., 2009). In the current study, members of *Actinobacteria* were determined to be the second most abundant in the bulk and rhizosphere soils of the two crops (buckwheat and barley). Gram-positive bacteria such as *Firmicutes* and *Actinobacteria* including *Bacillus, Micromonospora* and *Streptomyces* positively influence of plant growth, bioremediation activities as well as biocontrol (Trujillo et al., 2015). One of the unique species found only in barley bulk soil at Harrington was *Streptomyces vitaminophilus*, a species reported to produce halogenated antibiotics named pyrrolomycins (Mahan et al., 2016). However, more studies are needed to test the influence of this antibiotic producing bacterium on plant health and wireworm control. Moreover, *Streptomyces* have been reported to greatly influence the structure of microbial populations due to their ability to produce natural substances playing an important role as biocontrol agents by killing or suppressing plant pathogens (Vurukonda et al. 2018; Schmidt and Spiteller, 2017). For example, *Streptomyces aburaviensis* produces antibiotics to protect themselves from other bacteria (Schmidt and Spiteller, 2017; Jones et al., 2017). According to Katz and Baltz (2016), several antibiotic classes produced by different *Streptomyces* species including: macrolides (tylosin, spiramycin); aminoglycosides (neomycin, kanamycin,); β-lactams (cephamycin, carbapenems); tetracyclines (tetracycline, chlortetracycline, oxytetracycline); polyenes (candicidin, amphotericin B, nystatin); peptides (actinomycin); and chloramphenicol (Katz and Baltz, 2016). In our study, *Streptomyces aburaviensis* was found to be unique to buckwheat rhizosphere soils at the Cross Road location but its biological activity is not known to us and more study is still required to confirm this finding for a potential application in crop protection or the biomedical areas. In addition, *Streptomyces cacaoi* was found in buckwheat rhizosphere soils at the Cross Road location in 2014 and 2015. According to Yoon et al. (2012), this species has a potential role as an antagonistic bacterium that may function as a biological control agent against plant nematodes. Nonetheless, some *Streptomyces* species are known as plant pathogenic organisms such as *Streptomyces scabiei* (also known as *Streptomyces scabies*) (Vurukonda et al., 2018; Komeil et al., 2014; Leiminger et al., 2013), which were found only in the buckwheat rhizosphere soils at the Cross Road location in this

study. *Streptomyces scabiei* is known as a plant pathogenic bacterium causing potato common scab that decreases potato production and results in economic losses in many potato fields by producing toxins called thaxtomins (Komeil et al., 2014; Leiminger et al., 2013). This finding, if confirmed, is intriguing in the context of high scab pressure in potato growing areas where buckwheat is promoted as a rotation crop for wireworm control. Indeed, a previous study by Wiggins and Kindel (2005) showed a high scab disease incidence in potato after two years of green manure treatment as performed in the current study. On the other hand, a one-year green manure treatment of buckwheat and canola combined has been reported to reduce the incidence of common scab, increase potato yield, as well as increase the density and activity of *Streptomyces* toward different soil borne pathogens, weeds and nematodes (Larkin and Honeycutt, 2012; Laznik et al., 2014). Furthermore, several *Paenibacillus* species are able to promote crop growth by facilitating biological nitrogen fixation and producing antimicrobial compounds that control plant pathogens and insects (Grady et al., 2016). Our study also identified *Paenibacillus, Rhodococcus* and *Arthrobacter* in one or both crop root system soils, but their specific roles in scab control or soil health remains to be clarified.

Three OTUs were found at the species level to be unique to the buckwheat rhizosphere soils at both the Harrington and Cross Road (Stratford) locations. These species were *Methylophilusflavus, Saccharopolyspora tripterygii* and *Deinococcus yunweiensis* and they have been previously characterized as non-pathogenic bacteria (endophytic or symbiotic) (Gogleva et al., 2010; Li et al., 2009; Zhang et al., 2007). They have been reported to have a potentially beneficial effect on host plants via supporting plant growth under biotic and abiotic stress. Consequently, they were suggested for use in sustainable agricultural practices (Santoyo et al., 2016; Udwary et al., 2011; Trujillo et al., 2015; Kandel et al., 2017).

Since soil microbial diversity is reported to be strongly influenced by plant species and microbial characteristics from season to season (Siles and Margesin, 2017), we performed a two-year crop rotation using buckwheat and barley. The study showed that buckwheat contributed to reduce microbial load by 20% and 32% at the family and genus level, respectively, over two growing seasons compared to barley (13% and 18% at the same taxonomic levels). A higher soil microbial community was however observed in barley compared to buckwheat, which is consistent with previous findings that indicated higher populations of microorganisms observed after

planting barley, canola, and sweet corn crops (Larkin and Honeycutt, 2005). These microorganisms are beneficial to plants, such as *Pseudomonas* spp. and *Trichoderma* spp. (Larkin and Honeycutt, 2005). In this study, beneficial microorganisms were observed in barley and buckwheat, and their beneficial effect on the crops have already been reported by other studies (Trujillo et al., 2015; Sharma et al., 2005). According to Steinberga et al. (2012), soil microbial diversity plays an essential role in enhancing soil health as well as facilitates sustainable agricultural practices. In this context, rotation crops have been reported to alter the microbial diversity (Steinberga et al., 2012) as shown in this study. It has also been previously reported that buckwheat is an essential rotation crop and is known as a natural fertilizer. It is also associated with reducing plant diseases as well as supporting soil microbial diversity (Steinberga et al., 2012; Rancâne et al., 2009). Our study identified some unique species including *Streptomyces vitaminophilus* and *Streptomyces aburaviensis* considered as antibiotic producers. Thus, our findings support the beneficial role for buckwheat as a rotation crop in terms of reduction of bacterial loading. However, how this reduction is achieved from a mechanistic perspective requires further study.

To address the second objective of this study, which aimed to evaluate a potential correlation between the influences of a crop's rhizosphere diversity on wireworm density, a pilot wireworm trapping survey was performed to assess the incidence of wireworms following buckwheat and barley as rotation crops. The study showed that both crops contributed to reduce wireworm infestation over a two-year period. However, to draw a firm conclusion, a more robust wireworm density study using buckwheat as cover or rotation crop is required to further investigate the exact contributing factors and mechanisms for wireworm density reduction by buckwheat and barley as rotation crops.

In the context of pest control, integrated pest management (IPM) has been reported as a pest control program that integrates all available tools to protect agricultural crops from insects, weeds and diseases by reducing pest populations to a tolerable level in the most economic and environmentally friendly context (Dee Ann Benard, 2012). The current study constitutes an aspect of this IPM process and was intended to further our understanding of how the root system of rotation crops can influence the diversity and abundance of soil microbiomes.

4.2 General Conclusion

In conclusion, metagenomic 16S rRNA gene sequencing is well known as a powerful tool for the characterisation of microbial diversity in soils, and was used in the current study to investigate the microbial diversity in the rhizospheres of different rotation crops grown in wireworm infested and non-infested fields. The data showed that both buckwheat and barley rhizospheres modulated the soil microbial diversity at the family, genus and species levels and were correlated with a reduction in wireworm density over two years. In particular, unique bacterial species were found to be associated with buckwheat rhizosphere soils, some of which have previously been purported as beneficial bacteria in sustainable agriculture. Whereas the current study highlighted some interesting findings, it also had limitations. In particular, species identification may not have been as complete as it should be in all samples, mainly due to the lack of sequencing depth, as well as some technical bias between sample preparation, library generation, and sequencing runs. Moreover, to draw a firm conclusion on wireworm density, more sampling with many replications and normalization at the field scale should have been planned in order to carry out appropriate statistical analysis. Despite these shortcomings, the findings of the study led to new research questions and opportunities as they relate to the biological functions of some bacterial species in the rhizosphere, the mechanism associated with wireworm reduction, and possibly the potential for isolating uncultivable bacterial species using plants as a host. Taken together, findings from this study are in general agreement with previous studies and further demonstrate the positive effects of buckwheat in reducing the bacterial load in agricultural fields. The study also detected bacterial species that may have crop protection or biomedical application. Taken together, the primary hypothesis was verified as the study demonstrated that buckwheat root system influences the microbiome structure in rhizosphere, which may affect the wireworm population. However, more studies are required to assess the mechanisms of action.

CHAPTER FIVE: Literature cited

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APPENDIX 1

Table A1. List of components used for DNA extraction

	Kit Catalog # 12955-4	Kit Catalog # 12955-12
Component	Amount	Amount
PowerSoil–htp Bead Plate (w/Square Well Mat)	4	12
PowerSoil –htp Bead Solution	340 ml	1020 ml
PowerSoil –htp Solution C1	45 ml	135 ml
PowerSoil –htp Solution C2	128 ml	385 ml
PowerSoil –htp Solution C3	106 ml	320 ml
PowerSoil –htp Solution C4	550 ml	2 x 825 ml
PowerSoil –htp Solution C5	120 ml	360 ml
PowerSoil –htp Solution C6	66 ml	200 ml
PowerSoil –htp Spin Plates	4	12
PowerSoil –htp 2 ml collection Plates	8	24
PowerSoil –htp 1 ml collection Plates	16	48
PowerSoil –htp 0.5 ml collection Plates	4	12
PowerSoil –htp Microplates	4	12
PowerSoil –htp Centrifuge Tape	24	72
PowerSoil –htp Sealing Tape	16	48
PowerSoil –htp Elution Sealing Mats	4	12

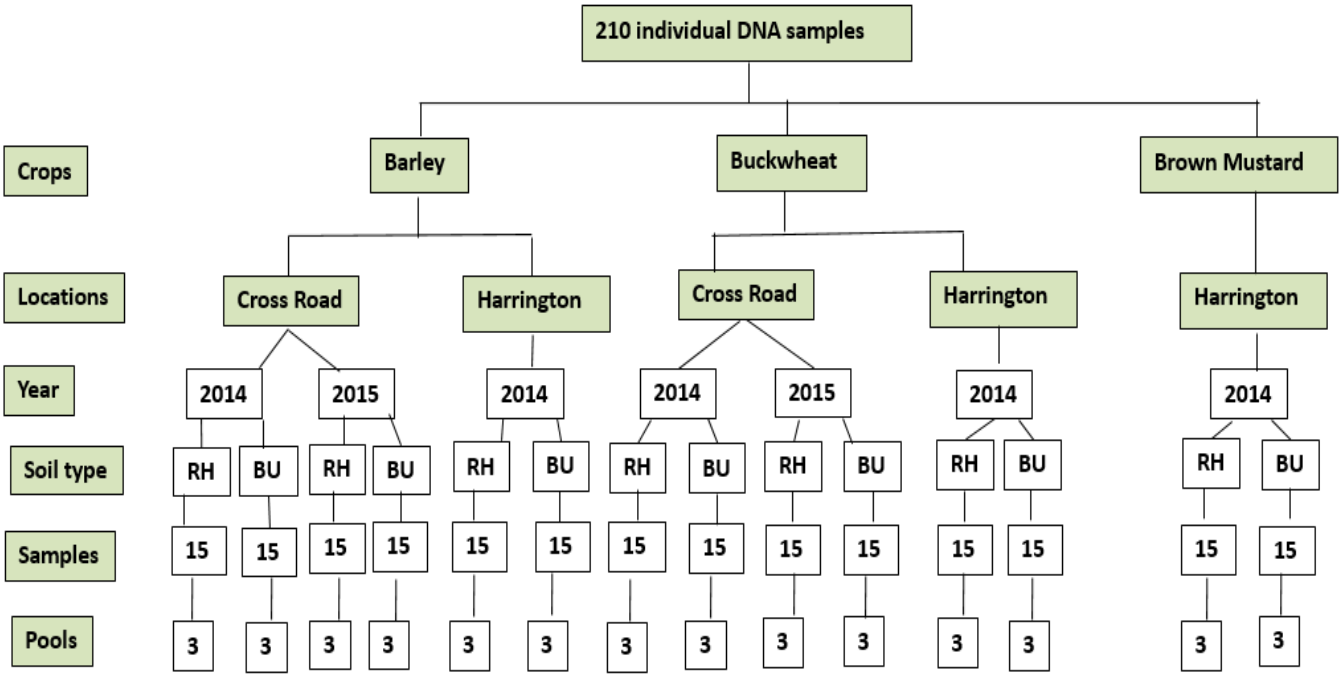

Figure A1. Sampling design, the number of samples for DNA Extraction and the number of pools in 2014 and 2015. RH, rhizosphere; BU, bulk.

APPENDIX 2

Table A2. The total number of sequences per sample

Year	Sample name	Total reads	Num. valid reads	Mapped reads	Unmapped reads
2014	BMHRH1	145,144	78,070	24,526	1,245
	BMHRH2	442,563	217,767	63,568	7,462
	BMHRH3	186,918	91,124	14,937	5,002
	BMHBU1	343,469	135,727	24,091	8,10
	BMHBU2	610,623	335,042	119,682	6,584
	BMHBU3	354,213	166,671	31,231	3,084
2014	BAHRH1	289,936	148,453	45,349	1,924
	BAHRH2	235,657	108,864	20,545	1,491
	BAHRH3	214,195	106,548	20,640	5,92
	BAHRH4	417,566	206,709	55,209	3,230
	BAHBU1	419,628	890,72	23,483	6,74
	BAHBU2	370,097	211,805	74,358	2,419
	BAHBU3	267,741	126,306	25,246	7,15
	BAHBU4	267,166	125,519	21,517	2,365
2015	BACRRH1	350,203	235,185	81,664	6,357
	BACRRH2	481,549	773,55	15,226	2,85
	BACRRH3	362,788	207,203	55,130	5,593
	BACRBU1	285,056	184,234	46,343	7,973
	BACRBU2	351,598	200,317	42,379	3,871
	BACRBU3	307,354	182,718	41,524	4,834
2014	BACRRH1	322,068	148,663	28,874	2,035
	BACRRH2	365,445	197,955	69,156	4,400
	BACRRH3	1,247,112	513,384	16,9371	7,404
	BACRRH4	336,834	185,084	50,120	3,728
	BACRBU1	303,394	163,082	33,388	1,235
	BACRBU2	253,510	399,55	36,22	4,01

	BACRBU3	274,328	169,581	58,725	4,336
	BACRBU4	689,589	460,951	187,461	14,060
2015	BWCRRH1	282,983	150,875	55,056	7,397
	BWCRRH2	258,304	137,740	45,898	3,856
	BWCRRH3	289,217	166,916	57,013	7,84
	BWCRBU1	652,391	118,403	36,108	2,72
	BWCRBU2	305,175	169,817	47,698	6,533
	BWCRBU3	314,439	164,317	37,885	1,339
2014	BWCRRH1	251,465	131,457	302,62	20,25
	BWCRRH2	322,419	146,008	408,02	15,27
	BWCRRH3	358,063	177,260	430,43	41,58
	BWCRRH4	294,073	138,377	341,84	31,22
	BWCRBU1	415,135	208,239	454,16	27,78
	BWCRBU2	280,065	128,348	242,59	22,95
	BWCRBU3	282,950	153,478	376,84	23,11
	BWCRBU4	251,199	154,676	425,89	40,71
2014	BWHRH1	260,776	143,013	371,45	10,33
	BWHRH2	989,097	448,925	123,737	47,04
	BWHRH3	250,823	140,502	329,63	10,01
	BWHRH4	268,108	131,176	184,42	13,13
	BWHBU1	314,941	184,340	423,63	20,13
	BWHBU2	666,639	350,996	104,437	43,18
	BWHBU3	304,239	161,453	428,05	11,79
	BWHBU4	259,049	136,964	238,19	22,74

BA = barley; BW = buckwheat; BU = bulk; RH = rhizosphere; CR = Cross Road; HR = Harrington.

APPENDIX 3

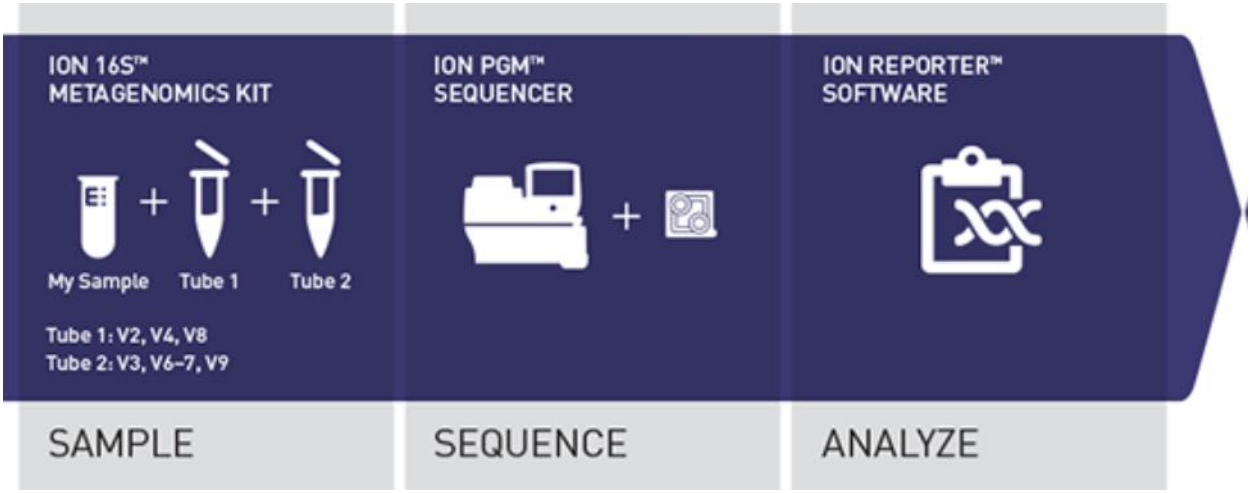

Figure A2. Ion 16S Metagenomics Workflow. The two primer pools included in the kit are used to amplify 16S variable regions from samples. After generating amplicons, the Ion Plus™ Fragment Library Kit was used to ligate barcoded adapters and synthesize libraries. Barcoded libraries from all 15 samples were pooled and templated on the OneTouch2™ system followed by 400 bp sequencing on the Ion PGM. Automated analysis, annotation and taxonomic assignment occur via the Ion Reporter Software pipeline. Classification of reads is achieved through alignment to either the curated MicroSEQ ID or curated Greengenes databases.

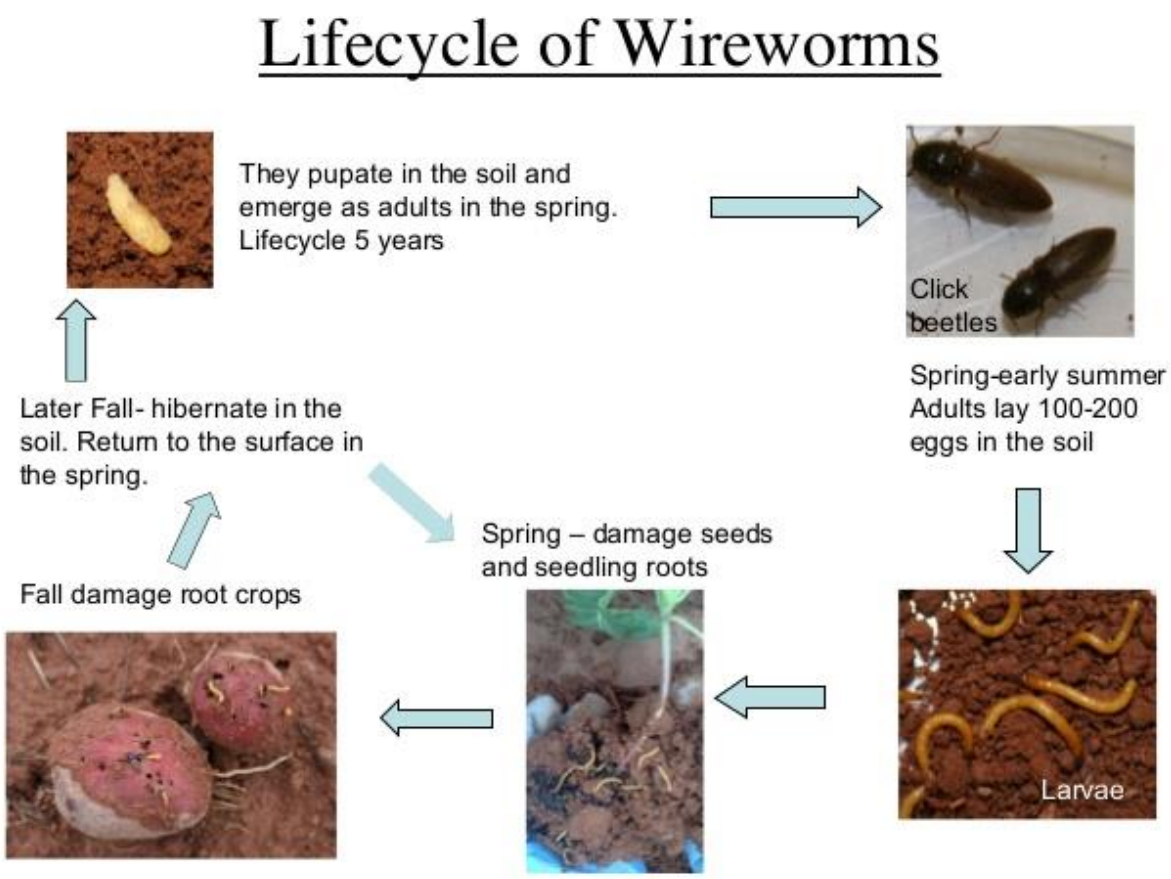

Figure A3. The life cycle of wireworms.

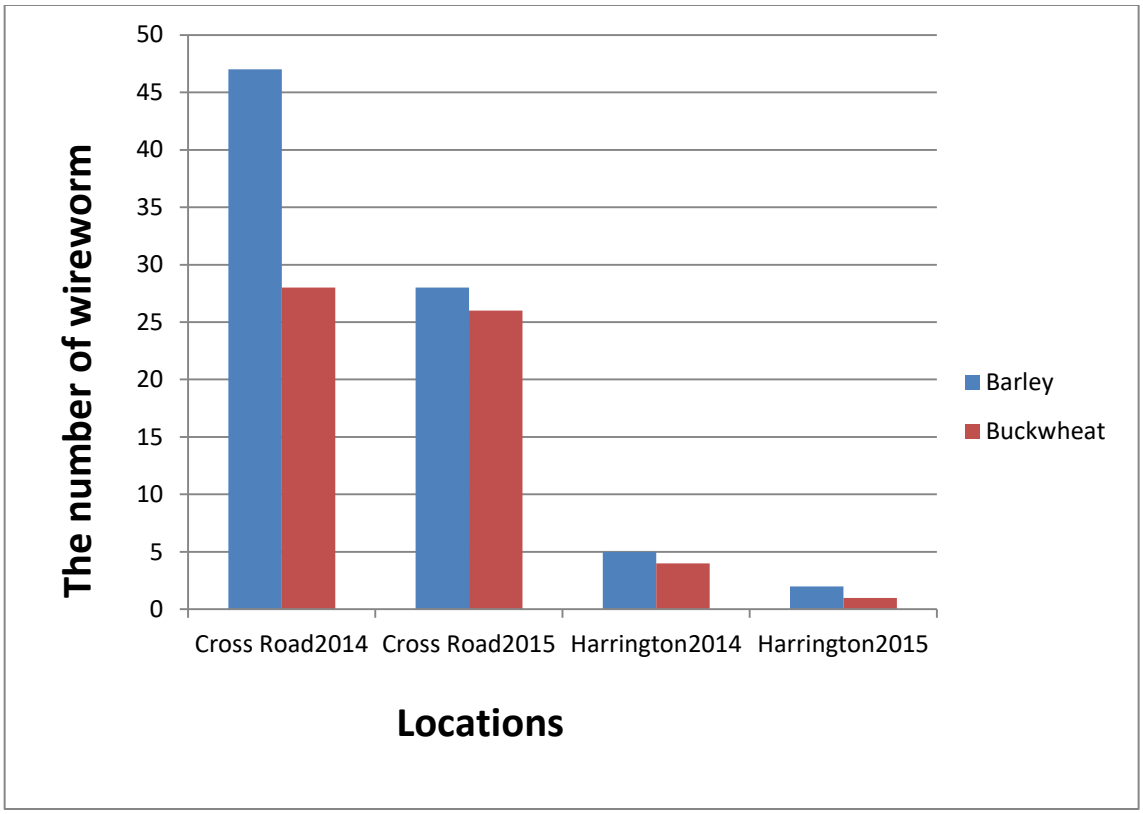

Figure A4. Comparison of wireworm counts from barley and buckwheat fields at both locations (Harrington and Cross Road).