

**ASSESSING THE ROLE OF PROBIOTICS FOR THE  
ENHANCEMENT OF SOIL QUALITY UNDER COVER CROPS**

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by  
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**ASSESSING THE ROLE OF PROBIOTICS FOR THE ENHANCEMENT OF  
SOIL QUALITY UNDER COVER CROPS**

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## ABSTRACT

Use of agro-chemicals over the past few decades has increased land productivity, however, frequent use of agro-chemicals may result in some negative impacts on the environment and soil microbial biodiversity. Use of alternative management such as application of probiotics for soil and plants is believed to promote soil biodiversity and soil nutrient cycling. Probiotics are believed to improve plant growth, root development and production of plant growth promoting substances. The main objective of this study was to quantify the effects of SCD Probiotics (Bio-Ag) on soil quality (SQ; microbial biomass, microbial communities, enzymatic activity) in association with cover crops in field and greenhouse studies.

This research was conducted at the Natural Resources Conservation Service (NRCS) Soil Health Demonstration Farm, Chariton County, Missouri to quantify probiotic effects on soil biology and enzyme activity. Prior to 2012, the site was comprised of conventional corn (*Zea mays* L.)-soybean (*Glycine max* L.) rotation with tillage and chemical fertilizer (anhydrous) use. Soils at the study site were Armstrong loam (fine, smectitic, mesic Aquertic Hapludalfs). The treatments included: control, treatment 1 (Trt1; 60L ha<sup>-1</sup> yr<sup>-1</sup> of Bio-Ag probiotics), treatment 2 (Trt2; 90L ha<sup>-1</sup> yr<sup>-1</sup> of Bio-Ag probiotics) and treatment 3 (Trt3; 120L ha<sup>-1</sup> yr<sup>-1</sup> of Bio-Ag probiotics) with three replications. Two equal split soil applications of probiotics were applied in September 2013 and May 2014. Soil samples were collected in August 2013, September 2013 and June 2014 from 0-6cm depth. Soil microbial biomass and community structures were analyzed using phospholipid fatty acid (PLFA) analysis. Standard enzyme assays were

used to analyze  $\beta$ -glucosidase, fluorescein diacetate hydrolase (FDA), dehydrogenase (DHA) and  $\beta$ -glucosaminidase activities. Total fungi biomass was highest in Trt3 followed by control, Trt1 and Trt2. Saprophytic fungi, protozoa and rhizobia biomarkers were significantly higher in Trt3 than control. Principal component analysis (PCA) revealed that PC1 and PC2 accounted for 62% of total variance. PCA also revealed that with time saprophytic fungi, protozoa and rhizobia biomass increased in Trt3 treatment. DHA ( $p<0.001$ ) and FDA ( $p<0.037$ ) were significantly higher in Trt3 than control, Trt1 and Trt2. No significant differences were found in total bacteria biomarkers of PLFA,  $\beta$ -glucosaminidase and  $\beta$ -glucosidase activity in field study. However, very strong positive correlations were found between all microbial groups and  $\beta$ -glucosaminidase activity in Trt3. Increasing trends in the values of soil fungal communities, rhizobia, DHA,  $\beta$ -glucosaminidase and FDA with probiotic concentration imply that probiotics can be used to improve SQ parameters.

A greenhouse study was conducted with Bio-Ag probiotics treatments including control, treatment 2 (Trt2; 90L ha<sup>-1</sup> yr<sup>-1</sup>), treatment 3 (Trt3; 120L ha<sup>-1</sup> yr<sup>-1</sup>) and treatment 4 (Trt4; 150L ha<sup>-1</sup> yr<sup>-1</sup>) using undisturbed soil cores (2356cm<sup>3</sup>) collected in PVC tubes in July 2014 from the buffer area adjacent to the field study site. The cores were seeded with hairy vetch (*Vicia villosa* Roth.) cover crops and placed in the greenhouse. Significant differences ( $p<0.10$ ) were observed between Trt3 and other treatments for total microbial, total bacteria and saprophytic fungi biomass. DHA was significantly higher in Trt3 than control under greenhouse study. Our study implied that probiotic application had a positive effect on microbial population including fungi and total microbial biomass at the of 120L ha<sup>-1</sup> yr<sup>-1</sup> rate in the greenhouse. Results also indicated a

strong positive correlation of DHA and FDA with all microbial groups respectively. Strong positive correlations were also found between  $\beta$ -glucosaminidase and all microbial groups.

A secondary study was conducted to quantify probiotic effects on hairy vetch roots and precursor-independent auxin production in soils in greenhouse study with control and treatment 3 (Trt3; 120L ha<sup>-1</sup> yr<sup>-1</sup> of Bio-Ag probiotics) treatments. Soil dilutions were plated on King's B medium selective for fluorescent pseudomonads. Fluorescent pseudomonads were significantly higher in Trt3 than control after the second probiotic application. Auxin production in soil samples was determined by high-performance liquid chromatography-Mass spectrometry (HPLC-MS/MS). Probiotics showed no effect on the precursor-independent production of auxin in soil samples. Two plant root samples (36 days old) with replicated images were also collected 7 days after the first probiotic application for scanning electron microscopy (SEM) observations. Images of SEM revealed more root hair growth and microbial colonization on hairy vetch roots treated with probiotic compared to control. Positive effect was observed on the root hairs growth of hairy vetch.

In the greenhouse study, with the highest concentrations of probiotics (150L ha<sup>-1</sup> yr<sup>-1</sup>), most of the microbial groups decreased including total microbial biomass. Our results suggested that probiotics can be used on cover crops to increase soil fungi biomass and DHA activity in the soils which are soil quality indicators. Furthermore, similar study can be conducted with high concentration of probiotics and non-treated control for the subsequent crops with addition of other parameters (soil physical and chemical properties, soil erosion, crop yield) to assess soil microbial activity and its relative effect

on other soil parameters and crop yield. Our secondary study implied that root hair growth and fluorescent pseudomonads are increased in hairy vetch with probiotic application. However, there was no effect of probiotic on auxin content in the soils. Our results also suggest that auxin-like substances can be detected using HPLC-MS/MS method and compounds can be recovered up to 87%.

# **CHAPTER 1**

## **INTRODUCTION**

Rapidly growing population of the world requires more food. Moreover, increasing population also reduces the arable land for agriculture. Agricultural scientists emphasize better use of soil to improve the crop production. To overcome this challenge, it is imperative to improve and maintain soil quality for sustainable agriculture.

To increase the productivity, the use of chemical fertilizers, pesticides and herbicides has also increased. However, excessive and frequent uses of these agrochemicals have polluted the environment to a great extent (Javaid, 2006). Over-application of chemical fertilizers can result in many negative effects such as leaching, pollution of water resources, destruction of beneficial microorganisms, crop susceptibility to diseases, soil acidification or alkalization or reduction in soil fertility thus causing irreparable damage to soil quality (Chen, 2006).

In addition, we are losing about 24 billion tons of topsoil worldwide each year due to soil erosion (Brown, 1995). Soil scientists have estimated that the United States has lost 30% of its topsoil in the past 200 years due to agricultural practices (Tyler et al., 1994). Environmental pollution, caused by excessive soil erosion and the associated transport of chemical fertilizers and pesticides to surface waters and groundwater has caused serious environmental and social problems throughout the world (Higa and Parr, 1994). Soil erosion not only inhibits vegetative growth, but reduces the biodiversity in soil as well (Pimentel et al., 1995). Moreover, surface runoff also poses serious threat to

aquatic life by contamination of water with chemicals (chemical fertilizers, pesticides, herbicides).

These issues have shifted scientific approaches towards alternative practices (Shaxson, 2006) that improve agricultural sustainability. In view of the economic and environmental problems of using chemical fertilizers, biofertilizers are considered as promising alternatives to chemical fertilizers and offer an option for a healthy environment (El-Ghandour and Galal, 1997). The application of microbial-based inoculants is considered very efficient and cost effective since it would maintain SQ substantially while reducing the use of chemical fertilizers and pesticides (Berg, 2009). Microbial-based inoculants can increase plant growth, speed up seed germination, improve seedling emergence, minimize external stress factors, protect plants from disease, and stimulate root growth by production of phytohormones (Bothe et al., 1992; Kloepper et al., 1980; Lugtenberg et al., 2002).

Probiotics are one example of microbial-based inoculants which may contain a single-species inoculant or complex cultures where numerous microbial species are grown together (Fuller, 1990; Gupta and Garg, 2009). Probiotics are defined as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller, 1989). Use of complex cultures has received more attention because they contain not only those microorganisms, which improve soil fertility but also species of beneficial microorganisms, which can suppress soil borne pathogens. (Fatunbi and Ncube, 2009).

Many complex cultured microbial-based inoculants are available on the market. One of those is SCD Probiotics (Bio-Ag) which consists of following species: *Bacillus*



*subtilis*, *Bifidobacterium animalis*, *Bacillus bifidum*, *Bacillus longum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Rhodopseudomonas sphaeroides*, *R. palustris*, *Saccharomyces cerevisiae* and *Streptococcus thermophilus*. Manufacturers claim positive effects of this product on many crops including rice (*Oryza sativa*), corn (*Zea mays*), wheat (*Triticum aestivum*) and vegetables (SCD, 2000, 2002a, 2013). Moreover, positive effects of the product reportedly include improved soil aggregate stability (SCD, 2002b).

Probiotics are used worldwide and have shown significant effects on human health (Isolauri et al., 1991), animal health (Kimura et al., 1983; Underdahl et al., 1982) as well as in aquaculture (Irianto and Austin, 2002). The use of probiotics in agriculture is very limited and only a few peer-reviewed research studies are available in the literature (Fatunbi and Ncube, 2009; Higa and Parr, 1994; Javaid, 2006).

In addition, cover crops are of crucial importance to maintain soil quality. Cover crops are usually planted to cover the soil during fallow periods which can minimize the land degradation. Cover crops provide several potential benefits such as improved tilth, protection of the soil against erosion, weed control, decreased soil compaction, building of soil organic matter as well as enhanced soil organism activity and diversity (Snapp et al., 2005). DuPont et al. (2009) have reported positive effects of cover crops on soil productivity as well as on soil fauna. One of the principal functions of cover crops is to prevent land degradation by erosion (Langdale et al., 1991) because accelerated soil erosion is often associated with deficient vegetative cover (Jenny, 1961; Lowdermilk, 1953). On the other side they compete with the main crops for soil moisture and

nutrients, serve as hosts for plant pathogens and insects (Martin and Bryson, 1997). Therefore, wise use of cover crop is essential to accomplish sustainable agriculture objectives (Langdale et al., 1991).

We hypothesize that the effects of SCD Probiotics along with cover crops can enhance soil microbial communities and soil enzymatic reactions, and thus enhance soil quality. Therefore, more extensive research is needed to understand the role of probiotics on soil microbial community and crop production under field and greenhouse studies.

Objectives of this study are:

- 1) To evaluate effects of SCD Probiotics (Bio-Ag) on soil quality biological indicators (microbial biomass, microbial communities, enzymatic reactions) under field conditions on cover crops.
- 2) To evaluate effects of SCD Probiotics on soil quality (microbial biomass, microbial communities, enzymatic reactions) under greenhouse study on hairy vetch.
- 3) To evaluate the effects of SCD Probiotics (Bio-Ag) on root growth of cover crops and precursor-independent microbial production of auxin.

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## **CHAPTER 2**

### **LITERATURE REVIEW**

#### **2.1. Soil Quality (SQ)**

According to Doran and Parkin (1994), SQ is defined as "the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation."

Soil functions can be physical, chemical and biological properties and processes. Evaluation of SQ as a whole is difficult, as one cannot measure all the properties and processes of a soil. However, to evaluate soil quality, certain indicators are used to assess the function of a soil. Those indicators could be physical (soil structure, aggregate stability, hydraulic conductivity, pore space), chemical (cation exchange capacity, pH, carbon content) or biological (microbial biomass, enzyme activities, mycorrhizal associations, microbial communities). Selection of indicators to measure SQ depends on the soil functions for which assessment is being made (Karlen et al., 1997).

The properties and processes of a soil are interconnected to each other. For example, enzymatic activity of a soil plays very important role in nutrient cycling, organic matter decomposition and play a substantial role in soil health and its environment (Das and Varma, 2011). Enzymes act as catalysts to perform specific biochemical reactions in a soil. Enzymes are broadly suggested as potential indicators of soil quality by (Dick et al., 1996; Frankenberger and Dick, 1983) because of their relationship to soil biology, ease of measurement and rapid response to changes in soil

management (Dick et al., 1996). However, it should be noted that assessment of any single enzyme does not indicate the SQ. Analysis of multiple specific soil enzymes along with other physical, chemical and biological properties and processes is necessary to assess the SQ.

Aggregate stability is an important feature of soil to maintain the soil structure. Soil structure is the three-dimensional arrangement of organic and mineral complexes (aggregates) and pore spaces (Rillig and Mummey, 2006). Aggregates are divided into two major sub categories on the basis of their diameter size; microaggregates ( $< 250 \mu\text{m}$ ) and macroaggregates ( $> 250 \mu\text{m}$ ). Microaggregates in a soil, which are formed by clay microstructures along with microbial and plant residues (Pokharel, 2013), are then bound together through binding agents (i.e fungal hyphae, polysaccharides, glomalin) to create macroaggregates (Six et al., 2004). Biological activities of a soil play an important role in the creation and maintenance of soil structure and aggregates.

Often nutrients in the soil are not in available form for plant utilization. Soil organisms play a central role in nutrient cycling in soils, making them available to plants, transforming some nutrient elements to gaseous forms which can be lost from soil, and other transformations which predispose nutrients to loss (Dixon and Tilston, 2010).

## **2.2. Microbial Based Inoculants**

The concept of microbial-based inoculants in agriculture is not new. Microbial inoculants are amendments to promote soil and plant health through the applications of beneficial microorganisms. Applications of inoculants can be an efficient and cost effective alternate to chemical fertilizers and pesticides, and there are now an increasing

number of inoculants being commercialized for various crops (Berg, 2009). Benefits of inoculants for plants could be attributed to following three main mechanisms: (i) PGPMs acting as biofertilizers (such as nitrogen-fixing bacteria and phosphate-solubilizing bacteria) assisting plant nutrient uptake by providing fixed nitrogen or other nutrients (Kennedy and Islam, 2001), (ii) Phytostimulators (microbes expressing phytohormones such as *Azospirillum*) can directly promote the growth of plants, usually by producing plant hormones (Glick et al., 2007), (iii) Biological control agents (such as *Trichoderma*, *Pseudomonas*, and *Bacillus*) protect plants against phytopathogenic organisms (Mohiddin et al., 2010). Probiotics are one of the types of microbial-based inoculants which may contain a single-species or multiple species grown together (Gupta and Garg, 2009).

### **2.2.1. Probiotics**

Probiotics are defined in various ways in the past (Fuller, 1989; Lilly and Stillwell, 1965; Parker, 1974). One of the definitions is “Probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host” (Salminen et al., 1999). Probiotics are used worldwide and have shown significant effects on human and animal health. According to Robinson and Thompson (1952), a *Lactobacillus acidophilus* supplement given to formula-fed infants was thought to improve weight gain. Isolauri et al. (1991) showed that oral rehydration that included a strain of *Lactobacillus casei* promoted recovery from acute diarrhea in children. Pigs have been successfully protected against diarrhea by the treatment of probiotics (Kimura et al., 1983). In aquaculture, they are also found to be beneficial (Irianto and Austin, 2002).



The applications of probiotics in agriculture can improve crop growth and yield by increasing photosynthesis, producing bioactive substances such as hormones and enzymes, controlling soil diseases and accelerating decomposition of lignin materials in the soil, stimulating the decomposition of organic wastes and residues, and thereby releasing inorganic nutrients for plant uptake (Javaid, 2006). The majority of the scientists who are engaged in promoting this technology have reported that plant growth is just as good or better and the quality of plant products is superior to conventional farming (Xu et al., 2000; Yamada and Xu, 2000). However, use of probiotics in agriculture is very limited and only a few peer-reviewed research studies are available.

Many research studies in the past have observed positive effects of complex cultures including: increase in plant performance, and enhance the efficacy and reliability of healthy effects on crops (Marimuthu et al., 2002). Many complex cultured microbial-based inoculants are available in the market these days. One of those is SCD Probiotics (Bio-Ag) which consists following species: *Bacillus subtilis*, *Bifidobacterium animalis*, *Bacillus bifidum*, *Bacillus longum* *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Rhodopseudomonas sphaeroides*, *R. palustris*, *Saccharomyces cerevisiae* and *Streptococcus thermophilus*.

Manufacturers claim positive effects of this product on many crops including rice (*Oryza sativa* L.), corn (*Zea mays* L.), wheat (*Triticum aestivum* L.) and vegetables (SCD, 2000, 2002a, 2013). Moreover, positive effect of the product has also been reported on aggregate stability of soils (SCD, 2002b). However, none of these studies are peer-reviewed. We hypothesize that the effect of SCD Probiotics can be positively

correlated with soil microbial communities and soil enzymatic reactions, and can enhance soil quality. Therefore, more extensive research is needed to understand the role of probiotics on soil microbial community and crop production under field as well as laboratory trials/greenhouse studies before recommendations are made to enhance crop production.

### **2.2.2. Plant growth promoting rhizobacteria (PGPR)**

The rhizosphere is the surface region of soil, which is directly influenced by roots of plants and associated microorganisms. Rhizosphere is much richer in bacteria than the other regions of soil (Hiltner, 1904). Root-colonizing bacteria which colonize plant roots and promote plant growth are known as plant growth promoting rhizobacteria (PGPR). PGPR plays an important role for plant nutrition by increasing N and P uptake by the plants, and playing a significant role as PGPR in the biofertilization of crops (Cakmakci et al., 2005). Several studies in the past have observed that PGPR improves plant's quality and quantity. Moreover, PGPR improve and maintain soil fertility and productivity as well. Significant effects of inoculation of PGPR species *Azospirillum*, *Bacillus*, *Pseudomonas*, and *Enterobacter* in maize, canola, wheat and other crops have been achieved both in laboratory and field trials (Glick et al., 1997; Sharma and Johri, 2003). Barbieri et al. (1986) found that inoculation of wheat seedlings with *Azospirillum brasilense* increased the number and length of lateral roots. It has been also observed that PGPR have the ability to convert essential elements from unavailable form to available through biological processes (Vessey, 2003). Wu et al. (2005) reported that microbial inocula *Bacillus megaterium* and *Bacillus mucilaginosus* not only increased plant growth,

but also improved nutritional assimilation of the plant (total N, P and K). Furthermore, PGPRs play a significant role in controlling pathogenic organisms (Whipps, 2001).

Increased growth and yields of potato (*Solanum tuberosum* L.), sugar beet (*Beta vulgaris* L.), radish (*Raphanus sativus* L.) and sweet potato (*Ipomoea batatas* L.) due to PGPR's inoculum have been reported (Farzana et al., 2009). Diazotroph bacterial inoculation significantly increases seed cotton yield, plant height and microbial population in soil (Anjum et al., 2007). The use of PGPR with P-enriched compost in an integrated manner improves the growth, yield and nodulation in chickpea (Shahzad et al., 2008).

PGPRs are commonly used as inoculants for improving the growth and yield of agricultural crops and offer an attractive way to replace chemical fertilizers, pesticides, and supplements (Ashrafuzzaman et al., 2009). The use of bio-fertilizer and bio-enhancer such as N<sub>2</sub> (nitrogen) fixing bacteria and beneficial microorganism can reduce chemical fertilizer applications and consequently lower production cost (Saharan and Nehra, 2011)

PGPR are used as bio-fertilizers and have proven beneficial effects on plant growth. The term biofertilizer is used in a broad sense; however it can be defined as a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003).

Inoculation of PGPRs have been shown to be very beneficial for plant growth and development (wheat, maize and other crops) in previous research (Barbieri et al., 1986; Glick et al., 1997; Sharma and Johri, 2003). Performance of PGPR is substantially

influenced by environmental factors including soil type (Egamberdiyeva, 2007). Other studies have also indicated the soil type as a dominating factor responsible for the diversity of bacterial populations associated with plant's roots (Chiarini et al., 1998; Latour et al., 1996).

One of the key roles of PGPR is the production of plant growth substances such as auxins and gibberellins (Chanway, 2002). Auxin is a class of plant hormones: the most common and well characterized is indol-3-acetic acid (IAA), which is known to stimulate both rapid (e.g., increases in cell elongation) and long-term responses in plants (Cleland, 1971). Glick et al. (1997) have observed the positive effect of strains of *Pseudomonas* on root and shoot elongation in canola (*Brassica napus* L.), tomato (*Solanum lycopersicum* L.) and lettuce (*Lactuca sativa* L.). Other studies have reported that inoculation of *Pseudomonas* improve plant development through production of plant growth regulators by microbes at the root interfaces, which also results in better absorption of water and nutrients from the soil (Wu et al., 2005; Zimmer et al., 1995).

Biological nitrogen fixation contributes  $180 \times 10^6$  metric tons  $\text{yr}^{-1}$  globally, out of which symbiotic associations' produce 80% and the rest comes from free-living or associative systems (Graham, 1988). The ability to reduce and derive such appreciable amounts of N from the atmospheric reservoir and enrich the soil is confined to bacteria and Archaea (Young, 1992). These include symbiotic nitrogen fixing ( $\text{N}_2$ -fixing) forms, namely *Rhizobium*, the obligate symbionts in leguminous plants and *Frankia* in non-leguminous trees, and non-symbiotic (free-living, associative or endophytic)  $\text{N}_2$ -fixing forms such as cyanobacteria, *Azospirillum*, *Azotobacter*, *Acetobacter diazotrophicus*, *Azoarcus* etc (Saharan and Nehra, 2011).

Phosphorus (P) is a major essential nutrient which is required by plants in large quantities. However, a large portion of soluble inorganic phosphate applied to the soil as chemical fertilizer is immobilized rapidly and becomes unavailable to plants (Goldstein, 1986). Therefore, phosphate solubilization is also very important to make P available for plant's uptake. The ability of some microorganisms to convert insoluble P to available form, like orthophosphate, is an important trait in a plant growth promoting bacteria for increasing plant yields (Chen et al., 2006). The rhizospheric phosphate utilizing bacteria, commonly known as phosphate solubilizing bacteria (PSB), could be a promising source for plant growth promoting agent in agriculture (Chaiham et al., 2008). The use of PSBs as inoculants has shown increases in uptake of P by plants (Chen et al., 2006). The mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids (Goldstein, 1995), which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpombrekou and Tabatabai, 1994).

Significant effects of PSBs on P availability as well as on quality and growth of plants have been observed by many research studies in the past. Isolates from the rhizosphere of soybean are found to solubilize P *in vitro* along with other plant growth promoting traits and increases the soybean growth (Cattelan et al., 1999). Bacterial isolates *Pseudomonas* sp. and *Azospirillum* sp. from the rhizosphere soil and root cuttings of black pepper (*Piper nigrum* L.) exhibit high phosphate solubilizing ability *in vitro* (Ramachandran et al., 2007). Phosphate solubilizing *Bacillus* spp. stimulate plant growth through P nutrition in wheat (Whitelaw et al., 1997), and increasing the uptake of N, P, K and Fe in rice (Biswas et al., 2000). It has been observed that the application of PSMs and

PGPRs together can reduce P application by 50% without any significant reduction of grain yield in corn (Yazdani et al., 2009). PSBs are also found to enhance vegetative growth and fruit quality and reduce the pollution of environment (Attia et al., 2009).

Among the heterogeneous and naturally abundant microbes inhabiting the rhizosphere, the Phosphate Solubilizing Microorganisms (PSM) including bacteria have provided an alternative biotechnological solution in sustainable agriculture to meet the P demands of plants (PGPR). Furthermore, phosphate solubilization is also performed by fungi such as *Aspergillus* and *Penicillium*. Among the bacterial genera PSBs belong to *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Flavobacterium* and *Erwinia* (Rodríguez and Fraga, 1999).

### **2.3. Fungi in Soils**

Fungi are eukaryotic organisms that include microorganisms such as yeasts, molds and mushrooms. In soils, fungi are of crucial importance in C and N cycling because of their ability to degrade complex substrates of plant origin which represent up to 90% of net primary productivity in most terrestrial ecosystems (Thorn and Lynch, 2007). Kingdom fungi includes; the Phyla Chytridiomycota, Zygomycota, Glomeromycota, Ascomycota, and Basidiomycota.

#### **2.3.1. Mycorrhiza**

Mycorrhizal symbiosis, a mutualistic plant-fungus association, is an essential feature of the biology and ecology of most terrestrial plants, since it influences their growth as well as their water and nutrient absorption and protects them from root diseases

(Smith and Read, 1997). Mycorrhizal fungi are a heterogeneous group of soil fungi that colonize the roots of about 240,000 plant species in nearly all terrestrial ecosystems and about 6000 species within the Zygomycota, Ascomycota, and Basidiomycota have been recorded as being mycorrhizal (Martin et al., 2007). Mycorrhizal associations are characterized by a bi-directional transfer of nutrients, where plants provide sugar to the fungi and these help the plants in the acquisition of mineral nutrients from the soil (Smith and Barker, 2002).

Additionally, mycorrhizal fungi also aid in soil-water extraction, increasing the drought tolerance of the host (Mathur and Vyas, 2000), improves the ability of a plant to tolerate heavy metal toxicity (Khan, 2001), attacks by pathogens (Fusconi et al., 1999) and herbivores (Borowicz, 1997).

Two types of mycorrhizal associations are known endo- and ectomycorrhizal. Endomycorrhizal fungi are characterized by extensive growth within (i.e, intracellular) cortical cells, but little extension into soil. These fungi produce extracellular enzymes that break down organic matter, enabling the plant to assimilate nutrients mineralized from organic compounds present in the colloidal material surrounding roots (Johnson, 2009). However, ectomycorrhizal fungi colonizes host plant's root extracellularly. Ectomycorrhizal fungi may produce large quantities of hyphae on the root and in soil. These hyphae function in the absorption and translocation of inorganic nutrients and water, but also release nutrients from litter layers by production of enzymes involved in mineralization of organic matter (Johnson, 2009). Moreover fungi stimulate microbial activities and promote enzyme activities in soil with a positive consequence for the

increases in plant growth through the enhancement of nutrient mineralization and availability (Li et al., 2012).

### **2.3.2. Arbuscular Mycorrhizal Fungi (Amf)**

Arbuscular mycorrhizal (AM) fungi are soil microorganisms that establish a mutual symbiosis with the majority of higher plants, providing a direct physical link between soil and plant roots (Strullu, 1991). The diagnostic feature of arbuscular mycorrhizae (AM) is the development of a highly branched arbuscule within root cortical cells. The fungus initially grows between cortical cells, but soon penetrates the host cell wall and grows within the cell (Johnson, 2009).

These fungi are the most ancient (Redecker et al., 2000) and widespread mycorrhizal associations. AMF were recently placed in a new monophyletic phylum, the Glomeromycota, encompassing three orders (Schussler et al., 2001) and five families (Morton and Redecker, 2001). Associations with these fungi are widespread among tropical trees, shrubs and herbs (Harley and Smith, 1983), including members of the Araucariaceae (Smith and Read, 1997). About 95% of the world's plant species belong to characteristically mycorrhizal families (Smith and Read, 1997) and potentially benefit from AM fungus-mediated mineral nutrition (Jeffries and Barea, 1994) due to the fundamental role played by these glomalean fungi in biogeochemical element cycling. AMF symbioses occur in almost all habitats and climates, including disturbed soils (Enkhtuya et al., 2002) and those derived from mine activities (Bi et al., 2003).

Arbuscular mycorrhiza (AM) represents an important ecosystem service in the capacity of soil to function as a vital system to sustain biological productivity and



environmental quality (Karlen et al., 1997). AM has the potential to promote plant nutrition and growth, and reduce nutrient leaching. Enhanced plant phosphorus (P) uptake is generally considered the main benefit of AM to plants (Abbott and Robson, 1984).

Furthermore, arbuscular mycorrhizal fungi have found to be very effective against heavy metals and other climatic stresses. It is reported that arbuscular mycorrhizal fungi may often lower Cd mobility and toxicity by increasing soil pH (Chen et al., 2004), sequestering Cd inside extraradical mycelium (Chen et al., 2004) and binding Cd to glomalin (González-Chávez et al., 2004). Glomalin is an insoluble glycoprotein synthesized and released by AMF (Wright and Upadhyaya, 1998), and may bind heavy metals in the soil (González-Chávez et al., 2004). Previous studies have also shown that glomalin production and the concentration of glomalin related soil protein would increase under unfavorable growing conditions such as heavy metal (Cornejo et al., 2008) and salinity (Hammer and Rillig, 2011) stresses.

Arbuscular mycorrhizal fungi (AMF) are naturally active under upland ecology and upland rice has been reported to be partially dependent on native AMF for P acquisition (Saha et al., 2006).

#### **2.4. Enzymes as indicators of soil quality**

Enzymes are proteins produced by living organisms that acts as catalysts to perform specific biochemical reactions. Likewise, in the soil they are known to play a substantial role in soil health and its environment (Das and Varma, 2011). In soils, enzymes are believed to be primarily of microbial origin (Ladd, 1978) but they also

originate from plants and animals (Tabatabai, 1994). They are usually associated with viable proliferating cells, but enzymes can be excreted from a living cell or be released into soil solution from dead cells (Tabatabai, 1994). The free enzymes complex with humic colloids and may be stabilized on clay surfaces and organic matter (Boyd and Mortland, 1990). Enzymes are broadly suggested as potential indicators of soil quality (Dick et al., 1996; Frankenberger and Dick, 1983) because of their relationship to soil biology, ease of measurement and rapid response to changes in soil management (Dick et al., 1996). However, it should be noted that presence of any single enzyme does not indicate the soil quality. Analysis of multiple soil enzymes and other chemical and physical properties is necessary to assess the soil quality efficiently. In addition, enzymes catalyze all the biochemical reactions and play a vital role in nutrient cycling (Bandick and Dick, 1999), stabilize soil structure, decompose organic wastes, aid in the formation of organic matter, hence playing an important role in agriculture (Dick, 1997; Dick et al., 1994).

#### **2.4.1. Dehydrogenase**

Dehydrogenase is an enzyme present in all soil microorganisms (Dick, 1997), and it does not live free in the soils (Frankenberger and Dick, 1983). This enzyme is exclusively intracellular, therefore its activity is considered to be an indicator of total microbial activity (Gil-Sotres et al., 2005; Schnurer and Rosswal, 1982). Dehydrogenase oxidizes soil organic matter by transferring protons and electrons from substrates to acceptors, therefore it plays an important role in respiration pathways of soil microorganisms (Das and Varma, 2011). Hence it does not live freely in the soils;

assessment of dehydrogenase can be used as an indication for soil microbial activity which is essential for maintaining soil quality. Dehydrogenase reflects total range of oxidative activity of soil microflora (Skujins, 1976).

Dehydrogenase activity (DHA) has been used as an indicator of soil microbial activity in variety of soils including Mediterranean arid soils (Garcia et al., 1994), humid regions of north-west Germany (Beyer et al., 1992) and Mediterranean forest soils of southern Spain (Quilchano and Marañón, 2002). DHA is usually greater at the surface soil (0-30cm) and declines with soil depth (Velmourougane et al., 2013). DHA in soil depends on the availability of soil organic carbon and organic matter (Zaman et al., 2002), which is greater at surface soil (0-30cm). Significant correlations have been observed between soil organic matter and enzyme activities (Leirós et al., 2000; Roldan et al., 2005; Wlodarczyke et al., 2002). Nannipieri (1994) observed increased DHA in zero-till soil due to larger proportions of microbial biomass and carbohydrate-C per unit of organic C.

DHA also responds to changes in soil quality factors such as soil pollutants, heavy metals (Hinojosa et al., 2004), herbicides (Felsot and Dzantor, 1995), plant protection chemicals (Chen et al., 2001) and insecticides (Shetty and Magu, 1998). Soil DHA is positively and significantly correlated with soil pH, water content, Ca, Mg and K (Quilchano and Marañón, 2002). Litter-fall inputs also favor the overall soil oxidative activity as the litter-fall undergoes decomposition, smaller and simpler organic molecules are leached from the litter layer into the surface soil horizon, as water-soluble organic matter, thus providing a labile organic substrate for soil microorganisms and better enzyme activities (Görres et al., 1998).

Li and Sarah (2003) and Nannipieri et al. (1990) have observed significant decrease of DHA with increasing aridity along a climatic transect in Judean Desert of Israel and positive effect of increasing water on DHA respectively. Quilchano and Marañón (2002) have also reported positive correlation between DHA and soil water content in autumn. Garcia et al. (1994) observed enhancement of DHA due to rainy season in south-east Spain. Furthermore, Görres et al. (1998) and Banerjee et al. (2000) have reported positive relation between DHA and soil water content in forest and grassland soils respectively. These findings suggest considerable sensitivity of enzyme activity to slight change in water availability (Sardans and Peñuelas, 2005). Moreover cropping system also affects DHA in soils (Bandick and Dick, 1999). Velmouroungane et al. (2013) have observed higher DHA in legume-based cropping systems than in cereal crops, cotton (*Gossypium hirsutum* L.) and sugarcane (*Saccharum officinarum* L.). Soils under crop rotations were reported to have higher concentrations of microbial biomass and enzyme activity than in mono-cropping systems (Miller and Dick, 1995). Khan (1970) observed higher soil enzymatic activities in crop rotation with highest in rotation of grains and legumes.

DHA is reported to change significantly due to many other soil properties including texture, pH, total organic C, clay content (Beyer et al., 1993) and soil aeration (Gliński et al., 2000).

#### **2.4.2. $\beta$ -glucosidase**

$\beta$ -glucosidases are enzymes which can be found extracellular as well as intracellular. They are produced by a variety of organisms (plants, animals, fungi and

bacteria; Esen, 1993) predominantly from soil microbial heterotrophs, in particular members of the mucorales (fungi), such as *Actinomucor* or *Mortierella* (Hayano and Tubaki, 1985).  $\beta$ -glucosidases may be excreted into soil solution or immobilized enzymes of microbial origin sorbed to clays or humic colloids (Knight and Dick, 2004). They are called 'abiotic' enzymes, a term coined by Skujins (1976) to describe enzymes of biological origin no longer associated with living cells. Busto and Perez-Mateos (1995) extracted humic compounds from the soil and showed that the extract contained as much as 50% of the total  $\beta$ -glucosidase activity of the soil.

Research studies have shown that fungi were the primary source of  $\beta$ -glucosidases (Busto and Perez-Mateos, 2000). However, Waldrop et al. (2000) found significant correlations between  $\beta$ -glucosidase activity and biomarkers for gram-positive and gram-negative bacteria. In contrast, they did not find significant correlations between fungal markers and  $\beta$ -glucosidase activity. Being extracellular and intracellular,  $\beta$ -glucosidase is characteristically useful as a soil quality indicator, and may give a reflection of past biological activity, the capacity of soil to stabilize the soil organic matter and detect management effect on soils (Bandick and Dick, 1999; Ndiaye et al., 2000).  $\beta$ -glucosidase is involved in the enzymatic degradation of cellulose (Turner et al., 2002), and produce glucose as a final product, which is an important C energy source of life to microorganisms in the soil (Esen, 1993). Major role of  $\beta$ -glucosidase in soils is involvement in the carbon cycling. It plays a crucial role in the C cycle of soils and the product of its enzymatic hydrolysis is important as an energy source for soil microorganisms (Bandick and Dick, 1999; Tabatabai, 1994). In addition, it carries out specific hydrolyses and catalyze reactions involved in the biogeochemical

transformations of C, N, P and S and is likely to be an essential component of any assessment of soil microbial activity and substrate mineralization (Taylor et al., 2001).

$\beta$ -Glucosidase enzyme is reported to be very sensitive to changes in pH and soil management practices (Acosta-Martinez and Tabatabai, 2000). Its sensitivity to changes in pH can be used as a good biochemical indicator for measuring ecological changes resulting from soil acidification in situations involving activities of this enzyme (Das and Varma, 2011).

Turner et al. (2002) have observed most of the variations in  $\beta$ -glucosidase activity are due to microbial C and total C. They also have observed close relationship between  $\beta$ -glucosidase activity and many other soil properties including microbial biomass, soil organic matter and soil texture. These findings suggest that  $\beta$ -glucosidase activity can be used as an index of soil quality (Stott et al., 2010) including physico-chemical and biological parameters of soil (Turner et al., 2002).

Many research studies have shown that  $\beta$ -glucosidase is the most abundant and easily detected of the three enzymes involved in cellulose degradation in soil, and is rarely substrate limited, thus making it ideal to examine the importance of physico-chemical controls on the turnover of soil organic matter (Eivazi and Tabatabai, 1988).

$\beta$ -Glucosidase activity has been found to be sensitive to soil management (Bandick and Dick, 1999; Dick et al., 1996) and has been proposed as a consistent indicator of soil quality (Bandick and Dick, 1999; Ndiaye et al., 2000).

With regard to the enzymes involved in the carbon cycle,  $\beta$ -glucosidase has been the most widely used in the evaluation of soil quality in soils subjected to different management procedures (Gil-Sotres et al., 2005). Such as organic fertilization can

increase the activity of this enzyme (Bandick and Dick, 1999). Piotrowska and Coper (2010) have reported positive correlation between  $\beta$ -glucosidase activity and farmyard manure fertilization, which is obvious because organic fertilization enhances microbial population, which is the main source of enzyme in the soil (Böhme and Böhme, 2006). Positive influence of farmyard manure fertilization on the organic carbon content and on accumulation, mineralization and humification of organic matter has also been observed by many research studies (Mackowiak and Zebrowski, 1999; Mercik et al., 2004). This also suggests that activity of  $\beta$ -glucosidase is mainly related to C content of a soil. Eivazi and Tabatabai (1990) reported the glucosidase activity is significantly and positively correlated with organic C content. Significant relationship between  $\beta$ -glucosidase activity and organic carbon content is also shown by (Bandick and Dick, 1999). Cover crops are also positively correlated with  $\beta$ -glucosidase activity (Bandick and Dick, 1999). However, no positive relationship was found between  $\beta$ -glucosidase activity and nitrogen fertilization (Piotrowska and Coper, 2010).

#### **2.4.3. Fluorescein Diacetate (FDA) Hydrolase**

The use of fluorescein esters as a measure of enzyme activity was first noted by Kramer and Guilbault (1964). Fluorescein diacetate (3',6'-diacetylfluorescein [FDA]) is used as a general indicator of soil hydrolytic activity, as it measures the activities of proteases, lipases and esterases that are all capable of cleaving the fluorogenic FDA (Dick, 1997). This colorless compound is hydrolyzed by both free (exoenzymes) and membrane bound enzymes (Stubberfield and Shaw, 1990), releasing a coloured end product, fluorescein. FDA is hydrolyzed by a number of different enzymes, such as

proteases, lipases, and esterases (Guibault and Kramer, 1964). The ability to hydrolyze FDA thus seems widespread, especially among the major decomposers, bacteria and fungi (Schnürer and Rosswal, 1982). Generally more than 90% of the energy flow in a soil system passes through microbial decomposers (Heal and McClean, 1975); therefore an assay which measures microbial decomposer activity will provide a good estimate of total microbial activity (Adam and Duncan, 2001).

FDA hydrolysis, like dehydrogenase activity, is regarded by some as a reliable measure of total microbial activity although, unlike the dehydrogenases, these enzymes can function outside of the cell and form stable complexes with soil colloids (Schnürer and Rosswal, 1982). Kremer and Li (2003) have observed higher FDA activity in soils under crop rotation with cover crops in a no till system as compared to monoculture with conventional tillage (corn).

In addition, FDA hydrolysis cannot be considered a specific measure of bacterial and fungal hydrolytic activity within soil as the reaction can be catalyzed by a range of other soil organisms, including algae and protozoa (Barak and Chet, 1986). However, it has been used to determine amounts of active fungi (Söderström, 1977) and bacteria (Lundgren, 1981) and to locate acetyl esterases in living protist cells (Medzon and Brady, 1969). Moreover, the determination of FDA hydrolysis has the advantage of being simple, rapid, and sensitive, and thus a good indicator of SQ, especially for comparative studies of microbial activity in natural habitats (Adam and Duncan, 2001).

#### **2.4.4. $\beta$ -glucosaminidase**



N-acetyl-b-D-glucosaminidase (EC 3.2.1.30), also referred to as NAGase or  $\beta$ -glucosaminidase, is an enzyme that hydrolyzes N-acetyl-b-D-glucosamine (NAG), residues from the terminal non-reducing ends of chitooligosaccharides (Bielka et al., 1984). The substrates for this enzyme include chitobiose and higher analogs and glycoproteins (Parham and Deng, 2000). In soils, this enzyme participates in amino sugar production (Ekenler and Tabatabai, 2003) that eventually leads to production of mineral N (Dick et al., 2013).  $\beta$ -glucosaminidase is also found in human's lysosomes and cleaves the amino sugar N-acetyl-b-D-galactosamine (Parham and Deng, 2000). It is therefore also listed as  $\beta$ -hexosaminidase (EC 3.2.1.52) in Enzyme Nomenclature (Bielka et al., 1984). It was also identified in the cytosol of animal cells (Braidman et al., 1974). It belongs to one of the three chitinases that degrade chitin (Tronsmo and Harman, 1993). Chitin, which consists of NAG residues in b-1,4 linkages, is the second most abundant biopolymer on earth behind cellulose. Chitinases are found in bacteria, fungi, plants and invertebrates such as protozoans, arachnids, insects, crustaceans and nematodes and humans (Trudel and Asselin, 1989).

$\beta$ -glucosaminidase plays an important role in soil C and N cycling, in particular soil N mineralization (Acosta-Martinez et al., 2007; Ekenler and Tabatabai, 2002). Moreover,  $\beta$ -glucosaminidase is highly correlated with fungal biomass and is proposed to be used as a semi-quantitative indicator of soil fungal biomass (Miller et al., 1998). As a major structural component in insects and fungal cell walls, chitin is an important transient pool of organic C and N in the soil (Wood et al., 1994). Thus,  $\beta$ -glucosaminidase may play an important role in both C and N cycling in soil (Parham and Deng, 2000).

Activities of  $\beta$ -glucosaminidase are also involved in biological control of plant pathogens. Purified  $\beta$ -glucosaminidase from *Trichoderma* spp. demonstrated antifungal activities against several plant fungal pathogens (Lorito et al., 1994). It has long been recognized that suppression of plant pathogens in soil has been associated with the presence of *Trichoderma* spp. (Chet, 1987). Therefore,  $\beta$ -glucosaminidase in soil may suppress plant pathogenic fungi (Parham and Deng, 2000).

Activity of  $\beta$ -glucosaminidase may be sensitive to land use and agricultural management, such as tillage and residue management (Acosta-Martinez et al., 2007; Ekenler and Tabatabai, 2003; Udawatta et al., 2008). Due to the significant role of  $\beta$ -glucosaminidase activity in N transformations, it could be used as a potential indicator to assess the effects of compaction on N cycling (Pengthamkeerati et al, 2011).

## **2.5. Contribution of Cover Crops to Soil Quality**

Cover crops are usually planted to cover the soil during fallow periods including grasses such as wheat (*Triticum aestivum* L.), oat (*Avena sativa* L.), rye (*Secale cereale* L.), and ryegrass (*Lolium multiflorum* Lam.), and legume such as (*Vicia* sp.) and clovers (*Trifolium* sp.), and vetches (*Vicia* sp.) (Locke and Bryson, 1997).

Cover crops are of crucial importance to maintain soil quality. Cover crops provide several potential benefits such as improved tilth, protection of the soil against erosion, weed control, decreasing soil compaction, building soil organic matter as well as influencing soil organisms (Locke and Bryson, 1997). DuPont et al. (2009) has reported positive effects of cover crops on soil productivity as well as on soil fauna. One of the principal functions of cover crops is to prevent land degradation by erosion (DuPont et

al., 2009) because accelerated soil erosion is often associated with deficient vegetative land cover (Jenny, 1961). Erosion is one of the major problems for agriculture worldwide.

In 2010, it was reported that US is losing 982 million tons of soil per year and 740 million tons of soil per year by water and wind erosion respectively (USDA, 2013). Missouri is losing about 4.32 tons of cropland soil per acre annually due to water erosion (sheet and rill erosion) (USDA, 2013). However, if we compare between cultivated and non-cultivated land, erosion is more severe in cultivated land. Although no till conditions reduce the soil erosion heavy rains during fallow periods in summer and winter could still cause severe erosion. Accelerated soil erosion is often associated with deficient vegetative land cover (Jenny, 1961).

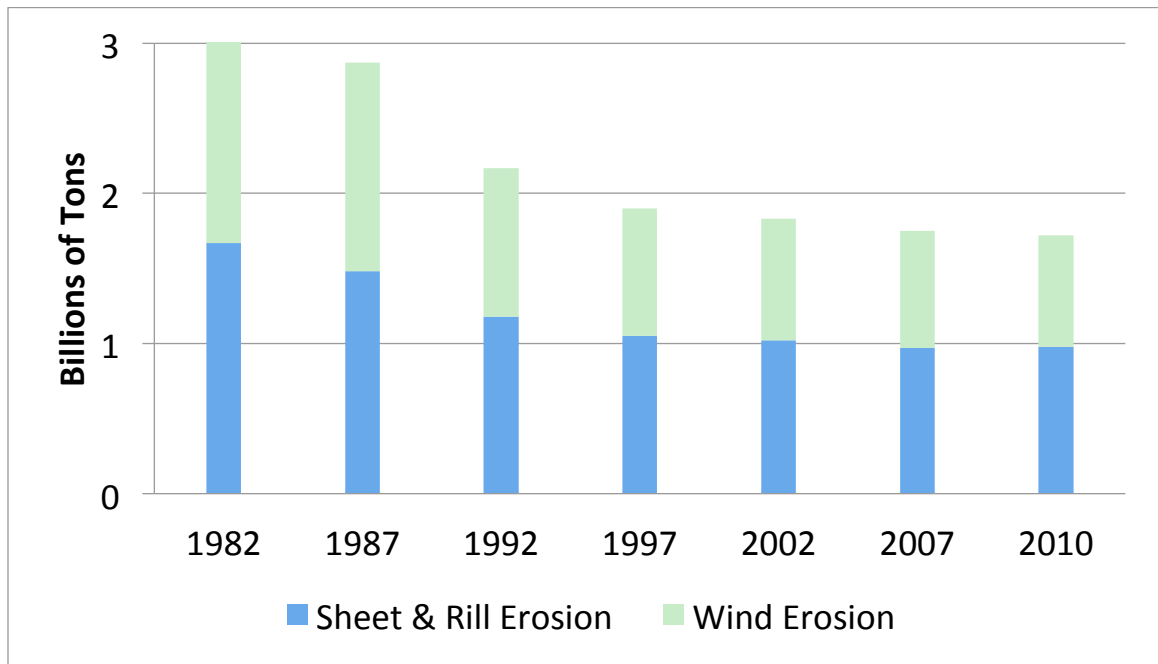


Figure 1.1. Overall soil erosion is 41% decreased in last three decades. Water (sheet and rill) erosion declined from 1.67 billion tons per year to 982 million tons per year, and erosion due to wind decreased from 1.38 billion to 740 million tons per year. (USDA, 2013).

Langdale et al. (1991) have observed that cover crops decreased soil losses by about 62%.

Winter crops are found to be very effective to control soil erosion. Zhu et al. (1989) compared no-till soybean (*Glycine max* L.) plots seeded to cover crops with a check-treatment without cover crops Udollic Ochraqualf in Missouri and found significant results. Mean annual soil losses from chickweed (*Stellaria media* L.), Canada bluegrass (*Poa compressa* L.), and downy brome (*Bromus tectorum* L.) treatments were decreased by 87, 95, and 96%, respectively, compared with the check plot with no cover crop (Langdale et al., 1991). Studies conducted in western Kentucky on a Typic Fragiudalf soil showed an 88% reduction of soil erosion for conventionally tilled soybeans planted following double-cropped wheat compared with conventional tillage without a cover crop (Langdale et al., 1991).

Winter cover crops may also reduce soil erosion (Smith et al., 1987) and NO<sub>3</sub>-N leaching during high rainfall periods in the winter (McCracken et al., 1994). In addition to improving soil characteristics and erosion control, they also provide N for corn crop in subsequent years. Locke and Bryson, (1997) has found due to cover crops nitrogen content of soil was increased which is significantly correlated with soil fauna. Planting winter cover crops after fall harvest increases cropping intensity and residue return and reduces the length of time the soil is left fallow.

The cover crops had small but significant effects on the SOC concentrations in the surface (0-15cm) or subsurface (15-30cm) depths when averaged across all sampling dates from May 1992 to April 1993 and from June 1993 to March 1994 (Kuo et al., 1997).

Cover crops are also found to have influence on soil organisms. On the other hand cover crops compete with the main crop for soil moisture and nutrients, serve as hosts for plant pathogens and insects (Martin and Bryson, 1997). Therefore, wise use of cover crop is essential to accomplish sustainable agriculture objectives (Langdale et al., 1991).

## **2.6. Microbial Production of Phytohormones (Auxin)**

Term plant growth regulators (PGRs) is used to define biologically active substances that regulate plant growth and influence physiological processes of plants at very low concentrations (Frankenberger and Arshad, 1995). Phytohormones are PGRs that are produced endogenously by plants. However, they can be synthesized by microorganisms when the required substrate is available. Therefore, under certain conditions, plants may not synthesize sufficient endogenous phytohormones for their optimal growth and development (Frankenberger and Arshad, 1995).

Some of the well-known phytohormones are auxins, gibberellins, cytokinins, ethylene, kinetin and abscisic acid. Barea et al. (1976) observed that among 50 bacterial isolates from rhizosphere 86% produced auxin and 90% produced kinetinlike substances. Auxin is one of the important phytohormones which play role in the plant growth and development. Chemical structure of an auxin is indole-3-acetic acid (IAA) which is considered as a very active auxin in plants which play role in cell enlargement, flowering promotion, fruit growth protein synthesis, root initiation, fruit ripening etc. (Frankenberger and Arshad, 1995). When the substrate is available, microorganisms in rhizosphere are more active in producing auxin than those from soil without root

(Strzelczyk and Pokojska-Burdziej, 1984). Tryptophan (TRP) is a precursor of auxin production by plants and microorganisms.

Conversion of TRP to IAA occurs through many routes including deamination, decarboxylation and hydrolysis (Frankenberger and Arshad, 1995). The conversion are catalyzed by aminotransferases, decarboxylase and oxidase (Liu et al., 1978). Microorganisms are found to produce IAA if TRP or TAM (a metabolite of TRP) is used as precursor (Dvornikova et al., 1970).

In vitro studies have also shown some microorganisms can produce small amounts of IAA in absence of precursor and possibly in natural conditions it could be synthesized in greater quantities (Smaly and Bershova, 1957). Many of the microorganisms are observed to produce IAA or Auxinlike substances without the addition of precursor including *Arthrobacter spp.* (Strzelczyk and Pokojska-Burdziej, 1984), *Bacillus spp.* (Ali et al., 2008; Müller et al., 1989), *Pseudomonas spp.* (Brown, 1972) and *Rhizobium spp.* (Ernstsen et al., 1987). Hartmann and Glombitza (1967) found that *Rhizobium leguminosarum* produced IAA from indole-3-acetonitrile which is not a metabolite of TRP. *Azotobacter spp.* are also found to produce IAA in the absence of TRP (Müller et al., 1989). However, addition of TRP stimulates IAA production (Romanow, 1965). Fallik et al. (1989) found that *Azospirillum brasilense* are capable of producing large quantities of IAA in the absence of TRP. Similar results were obtained by other studies too (Horemans et al., 1986). Interestingly, Müller et al., (1989) found that *Azospirillum lipoferum* produced more IAA in the absence of TRP than in the presence of TRP. Mycorrhizal fungi are also observed to produce small amount of IAA in the absence of TRP (Strzelczyk et al., 1992).

Auxin production has been shown to occur in the absence of TRP (Frankenberger and Arshad, 1995) but many of the studies have shown increase in IAA production in the presence of TRP (Lebuhn and Hartmann, 1993) since most of the microorganisms use TRP as an auxin precursor. Due to this reason many studies have focused on the auxin production in presence of precursor since in the absence of TRP, detectable levels of IAA are not found (Sarwar et al., 1992).

The only natural source of TRP for microorganisms is the root exudates (Frankenberger and Arshad, 1995). However, very few studies are conducted on the conversion of natural TRP to IAA through microbial activities since not all plants can release adequate amount of TRP (Martens and Frankenberger, 1993). Additions of TRP to soils enhance microbial production of IAA and positively affect the growth of plants. High concentrations of auxins could inhibit the root growth (Sarwar and Kremer, 1995) however, slow and continuous microbial produced auxin may have beneficial effects on plant growth (Frankenberger and Arshad, 1995).

## **2.7. Phospholipid fatty acid (PLFA)**

PLFA analysis can be used as an indicator of soil microbial biomass since it detects phospholipids which are essential membrane components of all living cells (Zelles, 1999). This is one of the advantages of PLFA analysis since it detects all the viable cells (except Archae) and phospholipid fatty acids, which are present in the cell wall of organism, degrade quickly upon an organism's death (White, 1988). Furthermore, using signature biomarker fatty acids, microbial communities can be characterized in major microbial groups such as fungi, gram-positive and negative bacteria, protozoa and

rhizobia. However, there are limitations in this method for characterizing microbial communities. For example, monounsaturated fatty acids are used as biomarkers for gram-negative bacteria, however it can also occur in gram-positive bacteria (Zelles, 1999). Linoleic acid, which is used as biomarker for fungi (Frostegård and Bååth, 1996) but it has been also observed that linoleic acid is also present in large concentrations in plants (Zelles, 1997). Therefore, Zelles (1999) has suggested linoleic acid as a good indicator of fungi when plants are not present in that system. Therefore, these biomarkers should be cautiously interpreted as they are not very specific for their characterized group (Zelles, 1997)

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**CHAPTER 3**

**EFFECTS OF PROBIOTICS ON SOIL MICROBIAL ACTIVITY,  
BIOMASS AND ENZYMATIC ACTIVITY UNDER COVER CROPS  
IN FIELD AND GREENHOUSE**

**3.1. Abstract**

Consumption of agro-chemicals over the past few decades has increased land productivity, frequent use of agro-chemicals have also resulted in some negative impacts on the environment and soil microbial biodiversity. Use of alternative management such as probiotics is believed to promote soil biodiversity and soil nutrient cycling. This research was conducted at the Natural Resources Conservation Service (NRCS) Soil Health Farm, Chariton County, Missouri to quantify probiotic effects on soil biological diversity and activity. Prior to 2012, the site was comprised of conventional corn-soybean rotation with tillage and chemical fertilizer (anhydrous) use. Soils in the study site were Armstrong loam (fine, smectitic, mesic Aquertic Hapludalfs). The Bio-Ag probiotics solution treatments included a non-treated control (2400ml of water), treatment 1 (Trt1; 144ml plus 2256ml water), treatment 2 (Trt2; 216ml plus 2184ml water) and treatment 3 (Trt3; 288ml with 2112ml water) with three replications. Two equal split soil applications of probiotics were applied in September 2013 and May 2014. Soil samples were collected in August 2013 (pre-treatment), September 2013 and June 2014 from 0-6cm depth. A greenhouse study was conducted using soil cores. Treatments in the greenhouse study included a non-treated control, treatment 2 (Trt2; 90L ha<sup>-1</sup> yr<sup>-1</sup>),



treatment 3 (Trt3; 120L ha<sup>-1</sup> yr<sup>-1</sup>) and treatment 4 (Trt4; 150L ha<sup>-1</sup> yr<sup>-1</sup>). Soil microbial biomass and community structures were analyzed using phospholipid fatty acid (PLFA) analysis. Standard enzyme assays were used to analyze  $\beta$ -glucosidase,  $\beta$ -glucosaminidase, fluorescein diacetate hydrolase (FDA), and dehydrogenase (DHA) activities. Total fungi biomass was highest in Trt3 followed by control, Trt1 and Trt2 in the field study. Saprophytic fungi, protozoa and rhizobia biomarkers were significantly higher in Trt3 than control. Significant differences were observed between Trt3 and other treatments in the greenhouse study for total microbial, total bacteria and saprophytic fungi biomass. In the field study, principal component analysis (PCA) revealed that PC1 and PC2 accounted for 62% of total variance. PCA also revealed that with time saprophytic fungi, protozoa and rhizobia biomass increased in Trt3. DHA and FDA were significantly higher in Trt3 than control, Trt1 and Trt2 in the field study. DHA was significantly higher in Trt3 than control under greenhouse study as well. No significant differences were found in total bacteria biomarkers of PLFA,  $\beta$ -glucosaminidase and  $\beta$ -glucosidase activity in field study. However, very strong positive correlations were found between all microbial groups and  $\beta$ -glucosaminidase activity in Trt3. Increasing trend in the values of soil fungal communities, rhizobia, DHA,  $\beta$ -glucosaminidase and FDA with probiotic concentration implies that probiotics can be used to improve soil quality (SQ) parameters.

### **3.2. Introduction**

Use of agro-chemicals over the past few decades has increased land productivity, frequent use of agro-chemicals has resulted in some negative impacts on the environment

and soil microbial biodiversity (Castillo et al., 2006; Javaid, 2006). Long-term studies have shown that applications of mineral fertilizers cause significant decline in soil microbial biomass and enzyme activity (Zhang et al., 2010). Thus, soil quality (SQ) is adversely affected due to the continuous use of agro-chemicals.

According to Doran and Parkin (1994), SQ is defined as "the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation." Use of alternative management approaches is necessary to promote and maintain SQ, specifically soil microbial biodiversity (Shaxson, 2006; Berg, 2009). Soil organisms play a central role in the recycling nutrients in soils, making them available to plants, transforming some nutrient elements to gaseous forms which can be lost from soil, and other transformations which predispose nutrients to loss (Dixon and Tilston, 2010). Thus, enhancing soil biological diversity helps promote SQ.

Microbial-based inoculants are widely used to enhance soil microbial activity (Deaker et al., 2004), probably due to less expensive production techniques compared to chemical fertilizers and pesticides. Researches have shown that amendments promote soil and plant health through the incorporation of beneficial microorganisms (Atieno et al., 2012; Cong et al., 2009; Mathu et al., 2012). Applications of these inoculants can be an efficient and cost effective alternative for reducing the use of chemical fertilizers and pesticides, and there are now an increasing number of inoculants being commercialized for various crops (Berg, 2009).

Probiotics are one of the forms of microbial-based inoculants, which may contain a single-species inoculant or complex cultures where numerous microbial species are

grown together (Gupta and Garg, 2009). The applications of probiotics in agriculture can improve crop growth and yield by increasing photosynthesis, producing bioactive substances such as hormones and enzymes, controlling soil diseases and accelerating decomposition of lignin materials in the soil, stimulating the decomposition of organic wastes and residues, and thereby releasing inorganic nutrients for plant uptake (Higa, 2000; Javaid, 2006). Scientists promoting this technology have reported that plant growth is just as good or better and the quality of plant products is superior to conventional farming (Xu et al., 2000; Yamada and Xu, 2000). However, use of probiotics in agriculture is very limited, as few research studies have been conducted to evaluate the significance on soil microbial community and biomass.

To evaluate SQ, certain indicators are used to assess the functions of a soil. Those indicators are based on physical (soil structure, aggregate stability, hydraulic conductivity, pore space), chemical (cation exchange capacity, pH, C content) and biological (microbial biomass, enzyme activities, mycorrhizal associations, microbial communities) soil properties. Selection of indicators to measure SQ depends on the land use and soil functions for which assessment is being made (Karlen et al., 1997).

Phospholipid fatty acid (PLFA) analysis is known to be a very reliable analysis to assess the microbial biomass and microbial community structures, since it detects the fatty acid components of phospholipids, which are essential membrane components of all living cells (Buyer and Sasser, 2012; Zelles, 1999). Since phospholipid-derived fatty acids degrade quickly upon death of organisms (White, 1988), PLFA analysis allow detection of only viable cells. Furthermore, using signature biomarker fatty acids, microbial communities can be characterized.

Enzymes in the soil are known to play an important role in soil health and its environment (Das and Varma, 2011). Enzymes are broadly suggested as potential indicators of SQ because of their relationship to soil biology, ease of measurement and rapid response to changes in soil management (Dick et al., 1996). However, it should be noted that analysis of multiple soil enzymes coupled with other chemical, physical and biological properties are necessary to assess the SQ reliably.

$\beta$ -glucosidases are one of the very important soil enzymes, which can be found extracellular as well as intracellular. Being extracellular and intracellular,  $\beta$ -glucosidase is a useful SQ indicator, and reflects past biological activity, the capacity of soil to stabilize the soil organic matter and management effect on soils (Ndiaye et al., 2000). Currently,  $\beta$ -glucosidase activity is included as a biological SQ indicator in models used in SQ assessment of soil management (Stott et al., 2010).  $\beta$ -glucosidase is involved in the enzymatic degradation of cellulose (Turner et al., 2002), and produces glucose as a final product, which is an important C and energy source for soil microorganisms (Esen, 1993).  $\beta$ -glucosaminidase enzymes participate in amino sugar production (Ekenler and Tabatabai, 2003) that eventually leads to production of mineral N (Dick et al., 2013). Activity of  $\beta$ -glucosaminidase can be sensitive to land use and agricultural management, such as tillage and residue management and could be used as a potential indicator of SQ (Acosta-Martinez et al., 2007; Udawatta et al., 2008; Pengthamkeerati et al., 2011).

The use of fluorescein esters as a measure of enzyme activity was first noted by Kramer and Guilbault (1963). Fluorescein diacetate (3',6'-diacetylfluorescein [FDA]) is used as a general indicator of soil hydrolytic activity, as it measures the activities of proteases, lipases and esterases that are all capable of cleaving the fluorogenic FDA

(Dick, 1997). FDA hydrolysis is regarded by some as a reliable measure of total microbial activity and it is often correlated with microbial respiration (Schnürer and Rosswall, 1982). DHA reflects total range of oxidative activity of soil microflora, and has been used as an indicator of soil total microbial activity. Dehydrogenase activity (DHA) has been used as an indicator of soil microbial activity in variety of soils including Mediterranean arid soils (Garcia et al., 1994), humid regions of north-west Germany (Beyer et al., 1992) and Mediterranean forest soils of southern Spain (Quilchano and Marañón, 2002). DHA is usually greater at the surface soil (0-30cm) and declines with soil depth (Velmourougane et al., 2013). It is reported to change significantly due to many soil properties including texture, pH, total organic C, clay content (Beyer et al., 1993) and soil aeration (Gliński et al., 2000).

The objective of this study was to evaluate the role of probiotics in promoting SQ by evaluating its effects on soil microbial biomass, microbial community structure (PLFA biomarkers) and soil enzymatic activities. This study was conducted at the Soil Health Demonstration Farm, Chariton County, Missouri, U.S.A which was established to assess the cover cropping for the improvement of SQ. Previously, field site was frequently used for corn (*Zea mays* L.)-soybean (*Glycine max* L.) rotation with tillage and frequent chemical fertilizer inputs. Therefore, we are evaluating the role of probiotics along with the cover crops for the betterment of SQ.

### **3.3. Materials and Methods**

#### **3.3.1. Study site, treatments, and soil collection**

The study site location was Soil Health Farm, Chariton County, MO, U.S.A (39°30'9.02"N, 92°43'13.66"W, Fig. 3.1). Soils in the study site are classified as Armstrong loam (Fine, smectitic, mesic Aquertic Hapludalfs), 5 – 9% slope, eroded. Baseline soil physico-chemical properties are listed in Appendix A. The study site included 12, 3m x 8m plots with 4 treatments in randomized block design. Experimental design was imposed on mixed cover crops which were already seeded. However, vegetation at the time of experimental design layout was in very early stage (Fig. 3.2 and 3.3).

Cover crops were established in July 2013 at the study area for soil quality improvement. Cover crop species included winter peas (*Pisum sativum* L.), cowpeas (*Vigna unguiculata* L.), hairy vetch (*Vicia villosa* Roth.), buckwheat (*Fagopyrum esculentum* L.), radishes (*Raphanus sativus* L.), crimson clover (*Trifolium incarnatum* L.), sorghum (*Sorghum bicolor* L.), oats (*Avena sativa* L.), turnips (*Brassica rapa rapa* L.), sunflower (*Helianthus annuus* L.), annual rye (*Lolium multiflorum* L.), sun hemp (*Crotalaria juncea* L.), cereal rye (*Secale cereale* L.), sweet clover (*Melilotus officinalis* L.) and triticale (*Triticosecale*) as a mixture on the study plots.

Split applications of probiotics were applied in September 2013 and May 2014 with a hand sprayer. The Bio-Ag probiotics solution treatments included a non-treated control (2400ml of water), treatment 1 (Trt1; 144ml probiotic solution plus 2256ml water), treatment 2 (Trt2; 216ml probiotic solution plus 2184ml water) and treatment 3 (Trt3; 288ml probiotic solution plus 2112ml water). The application of probiotics was based on 0, 60, 90, and 120L ha<sup>-1</sup> yr<sup>-1</sup> rates.

Probiotics used in this study (SCD Bio-Ag) contained following microbial species: *Bacillus subtilis*, *Bifidobacterium animalis*, *B. bifidum*, *B. longum*, *Lactobacillus acidophilus*, *L. bulgaricus*, *L. casei*, *L. plantarum*, *L. fermentum*, *Lactococcus lactis*, *Rhodopseudomonas sphaeroides*, *R. palustris*, *Saccharomyces cerevisiae* and *Streptococcus thermophiles* with concentrations of  $>3.0 \times 10^5$  cfu (colony-forming unit)  $\text{ml}^{-1}$  lactic acid bacteria and  $<1.0 \times 10^6$  cfu  $\text{ml}^{-1}$  yeast (SCD Probiotics, Kansas City, MO).

Soil samples were collected in August 2013, September 2013 and June 2014 from 0-6cm depth. From each plot five samples in a zig-zag pattern were collected and 3 composite samples were prepared for each replication for the analysis. Samples were placed in zip lock bags, labeled and stored in a temperature control room at 4°C before the analysis.

### **3.3.2. Greenhouse study, treatments, and soil collection**

Undisturbed 36 soil cores were collected in 10cm diameter, 30cm long ( $2356\text{cm}^3$ ) PVC tubes in July 2014. Soil cores were taken from an adjacent buffer area to the study site. PVC cores were placed on the soil surface and pushed into the soils using Gator mounted Giddings probe (Fig. 3.4). Once taken, soil cores were sealed from both sides with plastic caps. Duct tape was used so secure soils inside the tube and brought for the greenhouse study.

The greenhouse study included four treatments: non-treated control, treatment 2 (Trt2), treatment 3 (Trt3) and treatment 4 (Trt4) with three replicates of each. Probiotic

application was based on 0, 90, 120, and 150L ha<sup>-1</sup> yr<sup>-1</sup> rates in two split applications. Hairy vetch cover crop was seeded on July 07, 2014, with 4-6 seeds per soil core.

Soil samples (0-10cm) were collected in August, October, and December 2014, in three replicates. Samples were placed in labeled zip lock bags and stored at 4°C before the analysis.

### **3.3.3. PLFA analysis**

PLFA analysis was based on a protocol by Bligh and Dyer (1959) and Hamel et al. (2006). Fatty acids used as biomarkers for specific soil microbial group are listed in Table 3.1. Approximately 4g of moist sieved soil sample was placed in screw-cap test tube and analyzed according to the procedure described by Hamel et al. (2006). Samples were dried overnight in a centrifugal evaporator and dry weights were determined. Bligh-Dyer extractant solution (4ml) with internal standard was added to each test tube. Tubes were capped tightly and shaken to mix the soil with the solution. Tubes were sonicated for 10min in an ultrasonic bath. After centrifuging for 10min, 1ml of each chloroform and water were added. Tubes were vortexed and centrifuged again for 10min. The upper phase was removed by aspiration and lower phase (contains extracted lipids) was evaporated at 30°C. Samples were stored overnight at -20°C. Lipid classes were separated by solid phase extraction (SPE) using a 96-well SPE plate containing 50 mg per silica. Then transesterification was processed and samples were prepared for gas chromatography (GC) vials. Chromatographic peaks indicating fatty acids were identified using MIS Sherlock (MIDI, Inc., Newark, DE, USA.) software using PLFALD1 calibration table (Buyer and Sasser, 2012).



### 3.3.4. Enzyme Assays

$\beta$ -glucosidase activity measurement is based on the colorimetric determination of released *p*-nitrophenol when soil was incubated with *p*-nitrophenyl- $\beta$ -D-glycopyanoside (PNG) substrate (Eivazi and Tabatabai, 1988). One gram of sieved soil (<2mm) was placed in a 50ml Erlenmeyer flask. Toluene (0.2ml) was added and flasks were placed in a fume hood for 15min. Then 4ml of MUB (pH 6.0), and 1ml of PNG solution were added to all samples and were incubated at 37°C for 1hr. After that, 1ml of 0.5 M CaCl<sub>2</sub> and 4ml of 0.1 M THAM buffer (pH 12) were added and the content was filtered through Whatman No. 2V folded filter paper. The yellow color intensity developed in the filtrates was measured on spectrophotometer at 405nm wavelength. A calibration curve was prepared using working standards. A control solution was included for each sample and the same procedure was followed for controls but adding the substrate solution after termination of the reaction using THAM buffer (pH 12). Activity was expressed in  $\mu\text{g } p\text{NP g}^{-1} \text{ soil h}^{-1}$ .

Fluorescein diacetate (FDA) activity was measured by measuring the release of fluorescein after the soil was incubated with FDA as described by Green et al. (2006).

DHA measurement was based on Ghaly and Ben-Hassan (1993) assay method.  $\beta$ -glucosaminidase activity measurement was based on the colorimetric determination of released *p*-nitrophenol after the soil was incubated with *p*-nitrophenyl-*N*-acetylene- $\beta$ -D-glycopyanoside (PNNAG) substrate (Eivazi and Tabatabai, 1988).

### **3.3.5. Statistical analysis**

One-way ANOVA was used to for the analysis to test the differences in soil microbial communities and enzymatic activities at three different times. Each PLFA microbial group and enzyme was analyzed separately to evaluate the differences among treatments with time. Least significant difference (LSD) was performed to quantify significant differences and interactions among treatments for multiple comparisons. Diversity index was calculated by Shannon's Diversity Index method (Hughes et al., 2001). Each microbial group was treated as a species, amounts of fatty acid biomarkers belonging to each microbial group represent the count of microorganisms present in each group. Pearson correlation coefficient analysis was performed to evaluate the correlations between microbial groups and enzymes activity. All analysis was conducted using IBM SPSS Version 21 except for the Principal component analysis (PCA), which was performed on XLSTAT software.

## **3.4. Results**

### **3.4.1. Field study**

#### **3.4.1.1. PLFA**

Significant differences were observed for total fungi and saprophytic fungi biomass ( $p \leq 0.10$ ; Table 3.2). Using LSD it was observed that fungi biomass was significantly higher in Trt3 than control and Trt2 in June 2014. Comparison of mean values of total fungi biomass showed that the highest value was obtained in Trt3 (480.4 ng g<sup>-1</sup>) followed by control (443.9 ng g<sup>-1</sup>), Trt2 (384.2 ng g<sup>-1</sup>) and Trt1 (261.6 ng g<sup>-1</sup>) in September 2013. In June 2014, the highest mean value was obtained in Trt3 (713.4 ng g<sup>-1</sup>)

<sup>1</sup>) followed by Trt1 (523.8 ng g<sup>-1</sup>), control (435.4 ng g<sup>-1</sup>) and Trt2 (298.4 ng g<sup>-1</sup>). Results indicated a distinct trend of increasing total fungi biomass value in Trt3 along with the time. However, for the Trt2 and control treatments, mean value decreased in June 2014 as compared to September 2013 (Fig. 3.5).

The highest mean AMF value was observed in the control (154.5 ng g<sup>-1</sup>) followed by Trt3 (140.8 ng g<sup>-1</sup>), Trt2 (130.2 ng g<sup>-1</sup>) and Trt1 (95.31 ng g<sup>-1</sup>) in September 2013. However, in June 2014 the highest value was obtained for Trt3 (199.47 ng g<sup>-1</sup>) followed by control (136.3 ng g<sup>-1</sup>), Trt1 (128.9 ng g<sup>-1</sup>) and Trt2 (100.8 ng g<sup>-1</sup>). Although there was no significant difference between the treatments for AMF biomarkers, the results suggest that there is a distinct trend of increasing AMF biomass in Trt3 as compared to other treatments (Fig. 3.5).

For saprophytic fungi, the highest mean value was observed in Trt3 (339.64 ng g<sup>-1</sup>) followed by control (289.4 ng g<sup>-1</sup>), Trt2 (254.0 ng g<sup>-1</sup>) and Trt1 (166.2 ng g<sup>-1</sup>) in September 2013 with no significant differences among treatments. In June 2014, the highest mean value was obtained for Trt3 (514.0 ng g<sup>-1</sup>) followed by Trt1 (394.8 ng g<sup>-1</sup>), control (299.0 ng g<sup>-1</sup>) and Trt2 (197.6 ng g<sup>-1</sup>). Trt3 was significantly higher than control and Trt2, but no significant differences were found between Trt3 and Trt1 (Fig. 3.5).

Fatty acid biomarkers of rhizobia did not differ significantly in September 2013 (Fig. 3.6). However, in June 2014, Trt3 (98.05 ng g<sup>-1</sup>) was significantly higher than Trt1 (49.72 ng g<sup>-1</sup>), control (18.72 ng g<sup>-1</sup>) and Trt2 (12.00 ng g<sup>-1</sup>).

For fatty acid biomarker of protozoa no significant differences were found among treatments in September 2013. The highest mean value was obtained for Trt3 (45.16 ng g<sup>-1</sup>)

<sup>1</sup>) followed by Trt2 (39.58 ng g<sup>-1</sup>), control (36.61 ng g<sup>-1</sup>) and Trt1 (18.50 ng g<sup>-1</sup>). In June 2014, Trt3 (50.91 ng g<sup>-1</sup>) was significantly higher than Trt1, control and Trt2 (Fig. 3.6).

There were no significant differences found among treatments for other biomarkers used in PLFA, the same increasing trends were observed in most of the other biomarkers used in PLFA analysis for Trt3 including total biomass (Fig. 3.6).

#### **3.4.1.2. Soil enzyme activity**

Overall mean  $\beta$ -glucosidase activity was found to be the highest in Trt3 (5.19  $\mu\text{g } p\text{NP g}^{-1} \text{ soil h}^{-1}$ ) followed by Trt2, Trt1 and control (4.63  $\mu\text{g } p\text{NP g}^{-1} \text{ soil h}^{-1}$ ). However, the differences were not significant among treatments (Fig. 3.7).

FDA hydrolase activity did not differ significantly in August and September 2013 among treatments (Fig. 3.7). However, the highest FDA hydrolase activity was observed in Trt3 followed by Trt1, control and Trt2 in August; and Trt3 followed by Trt2, control and Trt1 in September 2013. In June 2014, Trt3 (8270 $\mu\text{g fluorescein g}^{-1} \text{ soil h}^{-1}$ ) was significantly higher than Trt2 (5770 $\mu\text{g fluorescein g}^{-1} \text{ soil h}^{-1}$ ), Trt1 (5260 $\mu\text{g fluorescein g}^{-1} \text{ soil h}^{-1}$ ) and control (5137 $\mu\text{g fluorescein g}^{-1} \text{ soil h}^{-1}$ ).

No significant differences were found among treatments for DHA in August 2013 (Fig. 3.8). In September 2013, DHA activity of Trt3 was significantly higher than Trt1 and Trt2 but the control. However, DHA was found to be significantly higher in Trt3 in June 2014 from all treatments.  $\beta$ -glucosaminidase activity was significantly higher in Trt3 than all the treatments in September 2013 (Fig. 3.8). In June 2014, Trt2 was significantly lower than all other treatments.

### 3.4.1.3. Pearson correlation coefficient

Strong correlations were observed among DHA,  $\beta$ -glucosaminidase activity and different microbial groups of PLFA analysis. Strong positive correlations were found for  $\beta$ -glucosaminidase activity with all of the microbial groups observed in Trt3 (Table 3.3). However, in the control treatment  $\beta$ -glucosaminidase was not positively correlated with any microbial biomarker (Table 3.4). Total microbial biomass had a strong positive correlation with DHA, total fungi, AMF, saprophytic fungi, and gram-positive bacteria, gram-negative bacteria, rhizobia, protozoa and actinomycetes biomass in Trt3. There was no positive correlation of total microbial biomass with any of the microbial groups in control treatment. Fungi to bacteria ratio (Fig. 3.9) was highly correlated with AMF, saprophytic fungi and diversity index in Trt3 than the control treatment. DHA was highly correlated with FDA in Trt3 but not in control. Saturated to unsaturated fatty acids ratio had a strong negative correlation with all microbial groups in Trt3. The control treatment also had a strong negative correlation with total fungi, AMF, rhizobia and protozoa. Diversity index had a strong positive correlation with all microbial groups in Trt3 but not in control.

### 3.4.1.4. Principal component analysis

PLFA data was analyzed for principal component analysis (PCA). PC1 and PC2 had eigenvalue of 9.99 and 4.21 and accounted for 43 and 18% of the total variance, respectively. Data from the Trt3 and control treatment were separated on the basis of sampling time (Fig. 3.10). Interestingly, Trt3<sup>S(September 2013)</sup> was found to be only in upper quadrants as compared to Trt3<sup>A(August 2013)</sup> and Trt3<sup>J(June 2014)</sup>. Trt3<sup>J</sup> loadings were found to

be on the lower left quadrant as compared to Trt3<sup>A</sup>, which was found on the lower right quadrant. Similar results were obtained for control treatment in C<sup>A</sup> and C<sup>J</sup> except one extreme value. Fatty acid biomarker scores were loaded to obtain the correlation of fatty acids with treatment and time. Most of the variance in the data for Trt3<sup>S</sup> was correlated with gram-negative (18:1 $\omega$ 7c), AMF (20:1 $\omega$ 9c) and actinomycetes (18:0-methyl) fatty acid biomarker. Positive correlation was found for PC1 loadings of Trt3 treatment with time ( $r^2 = 0.696$ ) but not in control ( $r^2 = 0.000$ ). Most of the variance in the data on PC2 was largely contributed by Trt3<sup>S</sup> (53.82%) followed by C<sup>S</sup> (38.16%).

### **3.4.2. Greenhouse study**

#### **3.4.2.1. PLFA**

Comparison of mean value of total biomass showed that the highest value was obtained in Trt2 followed by Trt3, Trt4 and control in October 2014 with no significant differences among treatments (Fig. 3.11 and Table 3.5). However, in December 2014, the highest mean value was obtained in Trt3 followed by Trt4, control and Trt2, with Trt3 significantly higher than control ( $p < 0.08$ ) and Trt2 ( $p < 0.07$ ). No other significant differences were observed for total microbial biomass (Table 3.6).

Total fungi biomass did not differ significantly among treatments in October 2014 with the highest mean value observed in Trt3 followed by Trt2, control and Trt4 (Table 3.5). In December 2014, Trt3 was significantly higher than Trt4 (Table 3.6). No other significant differences were found among treatment with the highest overall mean value observed in Trt3 followed by Trt2, control and Trt4 (Fig. 3.11)

Differences were found in total bacteria biomass among treatments in October and December 2014 (Fig. 3.11). Trt4 was significantly higher than control in October 2014. The highest mean value was observed in Trt4 followed by Trt3, Trt2 and control (Table 3.5). In December 2014, Trt3 was significantly higher than Trt2 and control (Table 3.6).

For protozoa biomass Trt2 was significantly higher than Trt4 in October 2014. No other differences were found among treatments (Fig. 3.12). Saprophytic fungi biomass was significantly higher in Trt3 than control and Trt4 in December 2014 only with highest mean value observed in Trt3 followed by Trt2, control and Trt4 (Table 3.6 and 3.7). Diversity index was observed to be significantly lower in Trt4 (1.39; 1.38) than Trt2 (1.60; 1.64) in October and August 2014 respectively.

#### **3.4.2.2. Soil enzyme activity**

$\beta$ -glucosidase activity was found to be highest in Trt3 ( $5.16 \mu\text{g } p\text{NP g}^{-1} \text{ soil h}^{-1}$ ) followed by Trt2 ( $5.03 \mu\text{g } p\text{NP g}^{-1} \text{ soil h}^{-1}$ ), control ( $4.86 \mu\text{g } p\text{NP g}^{-1} \text{ soil h}^{-1}$ ) and Trt4 ( $4.81 \mu\text{g } p\text{NP g}^{-1} \text{ soil h}^{-1}$ ) overall. Trt3 was significantly higher than all other treatments in December 2014. No significant differences were found among treatments in August and October 2014 (Table 3.7).

FDA hydrolase activity was found to be highest in Trt3 ( $5344 \mu\text{g fluorescein g}^{-1} \text{ soil h}^{-1}$ ) followed by control ( $5224 \mu\text{g fluorescein g}^{-1} \text{ soil h}^{-1}$ ), Trt4 ( $5058 \mu\text{g fluorescein g}^{-1} \text{ soil h}^{-1}$ ) and Trt2 ( $4912 \mu\text{g fluorescein g}^{-1} \text{ soil h}^{-1}$ ) overall. No significant differences were found among treatments in August and October 2014. However, control was

significantly higher than Trt3 ( $p < 0.07$ ), Trt2 ( $p < 0.005$ ), and Trt4 ( $p < 0.001$ ) in December 2014 (Table 3.8).

No significant differences were found among treatments for DHA activity in August and October 2014 (Fig. 3.13). In December 2014, Trt3 was significantly higher than all treatments and Trt4 was significantly lower than all treatments (Table 3.9).

$\beta$ -glucosaminidase activity in the soils among treatments was not significantly different during the study (Fig. 3.13 and Table 3.10). The highest value was obtained in control ( $2.81 \mu\text{g } p\text{NP } \text{g}^{-1} \text{ soil h}^{-1}$ ) followed by Trt3 ( $2.71 \mu\text{g } p\text{NP } \text{g}^{-1} \text{ soil h}^{-1}$ ), Trt4 ( $2.67 \mu\text{g } p\text{NP } \text{g}^{-1} \text{ soil h}^{-1}$ ) and Trt2 ( $2.52 \mu\text{g } p\text{NP } \text{g}^{-1} \text{ soil h}^{-1}$ ) overall.

### 3.5. Discussion

In our study we found that the probiotic, particularly Trt3 ( $120\text{L ha}^{-1} \text{ yr}^{-1}$ ), affected the soil microbial groups of total fungi and total bacteria positively in field and greenhouse studies, respectively (Fig. 3.5 and 3.11), which was also confirmed by Higa and Wididana (1991). Protozoa and rhizobia microbial groups also increased in Trt3 (Table 3.2 and Fig. 3.6). There were no significant differences in total bacteria including gram-positive, even though the probiotic solution claims to contain more gram-positive bacterial species than gram-negative. Similar results were observed for most of microbial groups in the greenhouse study. Total bacteria biomass increased to a great extent in the greenhouse study in Trt4 ( $150\text{L ha}^{-1} \text{ yr}^{-1}$ ), which implies that higher concentration of probiotics only promote bacterial population. In the greenhouse study at  $150\text{L ha}^{-1} \text{ yr}^{-1}$  probiotic dose, gram-positive bacteria were significantly higher than control, however,



the diversity index was decreased suggesting that probiotic doses higher than 120L ha<sup>-1</sup> yr<sup>-1</sup> decreased microbial diversity within a soil system.

Gram-positive bacteria strongly and positively correlated with diversity index in Trt3 compared with the control (Table 3.3 and 3.4). Diversity index describes the diversity of major microbial communities in the soils. The strong positive correlation of diversity index with most of the major microbial group including total microbial bacteria and fungal biomass in Trt3 treatment compared with the control indicated the presence of more diverse major groups as well as specific bacterial groups (gram-positive and gram-negative) due to probiotic applications, which has been previously confirmed (Higa and Wididana, 1991). Distinct increasing trends among values of total biomass at Trt3 suggest that higher concentrations of probiotics may affect changes in the different soil microbial groups. In the greenhouse study, the diversity index was found to be lower when probiotics were applied at 150L ha<sup>-1</sup> yr<sup>-1</sup>. This suggests that further increase in the dosage of probiotics repressed the diversity among different microbial groups in soils which could be due to the substrate competition between indigenous microorganisms and those introduced in the probiotic contents.

High correlation of diversity index with all microbial groups and DHA suggests the evenness of microbial communities, which is an indicator of healthy soils. However, diversity index can be only used for major microbial groups not for species diversity since it was calculated on the basis of microbial groups.

Total fungi biomass was significantly different between Trt3 and control treatment. Results imply that application of probiotic promoted indigenous soil fungal communities. Moreover, fungi to bacteria ratio has been used in the past to differentiate

between intensively disturbed agricultural systems and low-input organic farming systems (Bardgett and McAlister, 1999), and a higher fungi to bacteria ratio is considered an indicator of healthy soils. In the current study, the strong positive correlation of the fungi to bacteria ratio with diversity index and increasing trend of the ratio in Trt3 with time suggests that fungal communities were positively affected by the probiotic applications (Fig. 3.9). There was a 20% increase in fungi to bacteria ratio from August to September 2014 in Trt3. Moreover, in Trt3 greater positive correlation of fungi and protozoa could be attributed to food habits of fungi-protozoa combination of vampyrellids (a group of phylum Cercozoa) which is known to feed on fungi (Homma et al., 1979).

PCA results indicated that 18:1 $\omega$ 7c, which is used as an indicator of fungi, increased greatly in Trt3 treatment in Sep-2013. In contrast, the fatty acid 18:1 $\omega$ 9c, which is also used as a biomarker for saprophytic fungi (Zelles, 1997), did not increase. The latter has increased in Trt3 treatment in Jun-2014 (Fig. 3.10). However, 18:1 $\omega$ 9c has been reported to be found in plant tissues as well (Zelles, 1997). Trt3 had shown most of the variance affected by increase of gram-negative, actinomycetes and AMF in Sep-2013. However, protozoa, rhizobia and saprophytic fungi had not increased in Sep-2013 but increased greatly in Jun-2014. Increases in fungi can be justified by the presence of *Rhodopseudomonas palustris* in the probiotic contents, which is a photosynthetic bacteria, that secretes nitrogenous compound which is used as an important substrate for fungal communities (Condor et al., 2007).

In previous research studies,  $\beta$ -glucosaminidase highly correlated with fungal biomass and was considered a semi-quantitative indicator of soil fungal biomass (Miller

et al., 1998). A strong positive correlation of  $\beta$ -glucosaminidase with total soil fungi biomass in our study supports this observation. We could suggest that probiotics enhanced  $\beta$ -glucosaminidase activity and contribute to improved SQ.

Strong positive correlations were found between FDA activity and different soil microbial groups including protozoa and rhizobia. This result implies that FDA cannot be used as an indicator of specific microbial group since the reaction can be catalyzed by number of microorganisms including algae, bacteria and protozoa (Barak and Chet, 1986).

Changes in the soil fungal community could be due to the effects of probiotic on indigenous soil fungal groups, since no fungal organisms were present in the probiotic. Interestingly, similar results regarding unanticipated soil microbial community changes were also observed by Zhao et al. (2005). Other studies have also observed that soil fungal communities were largely affected by different soil organic amendments and management practices which suggested that soil fungal population is more sensitive to management practices as compared to other microbial groups (Wu, 2005).

PLFA analysis is widely used to evaluate soil microbial biomass and community structures. However, there are limitations in this approach for characterizing microbial communities. For example, monounsaturated fatty acids are used as biomarkers to characterize gram-negative bacteria, however it can also occur in gram-positive bacteria (less than 20% to the total PLFA content, Zelles, 1999). Linoleic acid, used as a biomarker for fungi (Frostegård and Bååth, 1996), is present in large concentrations in plants (Zelles, 1997). Therefore, Zelles (1999) had suggested linoleic acid as a good indicator of fungi when the plants are not present in the system. Therefore, these

biomarkers should be cautiously interpreted as they are not very specific for their characterized group (Zelles, 1997). Moreover, analysis of other soil biological activities can be included in the studies to test correlations between PLFA microbial groups and other biological parameters confirm the results obtained by PLFA analysis.

PLFA analysis has the advantage over various other techniques since microbial communities can be assessed without the selective isolation of individual microorganisms (Cavigelli et al., 1995). It has been widely tested to measure rapid changes in soil microbial community structure (Albers 1994; White et al., 1979). Also total PLFA amount is used as an indicator of total microbial biomass (Zelles et al., 1999) since the fatty acid biomarkers selected for analysis are widely present in microorganisms (Haack et al., 1994). Moreover, conventional cultured techniques would be inadequate to provide enough information about microbial communities since there are many known uncultured and unknown microorganisms present in the soil (Amann et al., 1995). PLFA is also advantageous since it provides the soil microbial community structure and diversity in situ as opposed to most probable number and viable plate count.

### **3.6. Summary and Conclusion**

Our results suggest that the concentration of probiotic (Trt3; 120L ha<sup>-1</sup> yr<sup>-1</sup>) had positive effects on soil fungal communities including both AMF and saprophytic fungi. Higher mean soil fungal community values were found in Trt3 than control. The highest mean value was observed for protozoa and rhizobia microbial group in Trt3. However, no significant results were observed in total bacterial community although the probiotic solution used in the study claimed to contain mostly bacteria (gram-positive and gram-

negative bacteria). Furthermore, in the greenhouse study bacteria biomass was increased greatly with the same treatment, which suggests the effect of probiotics varies when temperature and soil moisture is controlled. Increasing trends in soil fungal communities, rhizobia and protozoa among different probiotic treatments suggest that high concentrations ( $120\text{L ha}^{-1}\text{ yr}^{-1}$ ) of probiotics can be used in the future studies to further evaluate their effects on other soil microbial communities.

Results also indicated a strong positive correlation of DHA and FDA hydrolytic activity with all and various microbial groups respectively. Strong positive correlations were also found between  $\beta$ -glucosaminidase and all microbial groups. PCA showed that most of the variance in fatty acid biomarkers in high dose treatment at time one but rhizobia and protozoa fatty acid biomarkers increased at time two only.

With the highest concentrations of probiotics ( $150\text{L ha}^{-1}\text{ yr}^{-1}$ ), most of the microbial groups and total microbial biomass decreased. More soil biological parameters can be included in the future studies to further quantify the effects of probiotics on other soil biological parameters such as microbial biomass C, more enzyme assay methods and also to select select the most representative parameters to understand their effects. Furthermore, long-term experiments can be conducted to see the long term effects of probiotics on SQ and land productivity.

### 3.7. Tables and Figures

Table 3.1. Fatty acid biomarkers used for the microbial groups.

Microbial group	Biomarkers		
Gram positive	iso14:0	iso16:0	18:0 10-methyl
	15:0	16:0 10-methyl	iso19:0
	iso15:0	17:0	anteiso19:0
	anteiso15:0	iso17:0	
	17:0 10-methyl	anteiso17:0	
Gram negative	10:0 2OH	iso14:0 3OH	16:0 3OH
	10:0 3OH	15:0 2OH	16:1 2OH
	iso11:0 3OH	16:1 $\omega$ 7c	cyc17:0
	12:0 2OH	16:1 $\omega$ 7t	18:1 2OH
	12:0 3OH	16:1 $\omega$ 9c	18:1 $\omega$ 5c
	iso13:0 3OH	16:0 2OH	18:1 $\omega$ 7c
	cyc19:0 $\omega$ 9	cyc19:0 $\omega$ 8c	iso19:0
	cyc19:0 $\omega$ 6	anteiso19:0	
Arbuscular mycorrhizae (AMF) fungi	16:1 $\omega$ 5c	20:1 $\omega$ 9c	16:1 $\omega$ 11c
	22:1 $\omega$ 9c		
Saprophytic fungi	18:1 $\omega$ 9c	18:3 $\omega$ 3c	20:5 $\omega$ 3c
	18:2 $\omega$ 6,9c/18:0 ante	18:3 $\omega$ 6c	18:3 $\omega$ 6c(6,9,12)
		18:2 $\omega$ 6c	
Rhizobia	15:0 2OH	cyc19:0 $\omega$ 8c	
Actinomycetes	16:0 10-methyl	18:0 10-methyl	
	17:0 10-methyl		
Protozoa	20:2 $\omega$ 6,9c	20:4 $\omega$	20:4 $\omega$ 6c
	20:2 $\omega$ 6c	6,9,12,15c	20:3 $\omega$ 6c
		20:3 $\omega$ 3c	

Table 3.2. One-way ANOVA of microbial groups in control, treatment 1 (Trt1; 60L probiotic ha<sup>-1</sup> yr<sup>-1</sup>), treatment 2 (Trt2; 90L probiotic ha<sup>-1</sup> yr<sup>-1</sup>) and treatment 3 (Trt3; 120L probiotic ha<sup>-1</sup> yr<sup>-1</sup>) for samples collected in June 2014 at Chariton County Soil Health Farm, Missouri.

Community variable	F value	<i>P</i> -value
Total biomass (ng g <sup>-1</sup> )	1.335	0.329
Total fungi biomass (ng g <sup>-1</sup> )	3.125	<b>0.088*</b>
AMF <sup>†</sup> (ng g <sup>-1</sup> )	2.840	0.106
Gram positive (ng g <sup>-1</sup> )	1.365	0.321
Gram negative (ng g <sup>-1</sup> )	2.345	0.149
Saprophytic fungi (ng g <sup>-1</sup> )	3.278	<b>0.086*</b>
Rhizobia (ng g <sup>-1</sup> )	7.944	<b>0.009*</b>
Protozoa (ng g <sup>-1</sup> )	3.939	<b>0.054*</b>

\*Significant *p*-values in bold when alpha = 0.10

†AMF: Arbuscular mycorrhizae fungi.

Table 3.3: Overall pearson correlation coefficients (r-value) among soil variables for treatment 3 (Tr3; 120L probiotic ha<sup>-1</sup> yr<sup>-1</sup>) at Chariton County Soil Health Farm, Missouri.

Variables <sup>a</sup>	TMB	TF	AMF	Sap	TB	Gram+	Gram -	Rhi	Actin	Prot	F:B	Pr:Py	Mo:po	D. I	St:Ut	FDA	BGD	DHA
TF	.99**																	
AMF	.98**	.97**																
Sap	.98**	.99**	.95**															
TB	.98**	.96**	.99**	.94**														
Gram +	.99**	.99**	.99**	.98**	.98**													
Gram -	.99**	.99**	.99**	.98**	.98**	1**												
Rhi	.88**	.92**	.86**	.93**	.84**	.91**	.91**											
Actin	.96**	.95**	.97**	.93**	.98**	.95**	.95**	.80**										
Prot	.96**	.98**	.98**	.97**	.96**	.99**	.99**	.93**	.93**									
F:B	.90**	.94**	.84**	.96**	.82**	.90**	.90**	.91**	.81**	.90**								
Pr:Py	.93**	.96**	.92**	.97**	.90**	.96**	.96**	.94**	.87**	.97**	.96**							
Mo:po	-.88**	-.92**	-.85**	-.94**	-.83**	-.90**	-.90**	-.86**	-.83**	-.90**	-.97**	-.96**						
D. I	.94**	.97**	.92**	.98**	.89**	.95**	.95**	.95**	.88**	.96**	.98**	.99**	-.97**					
St:Ut	-.91**	-.93**	-.93**	-.92**	-.91**	-.93**	-.93**	-.77*	-.92**	-.92**	-.88**	-.93**	.93**	-.91**				
FDA	.53	.62	.56	.63	.50	.61	.61	.65	.48	.67*	.73*	.77*	-.78*	.73*	-.72*			
BGD	.53	.50	.46	.50	.46	.48	.48	.22	.49	.40	.55	.46	-.59	.47	-.64	.28		
DHA	.82**	.87**	.82**	.87**	.77*	.86**	.86**	.84**	.74*	.88**	.92**	.95**	-.95**	.93**	-.90**	.84**	.55	
BGM	.88**	.92**	.85**	.93**	.81**	.90**	.90**	.91**	.77**	.91**	.97**	.96**	-.94**	.96**	-.87**	.73**	.54	.96**

\*\* Significant at the  $P < 0.01$  level.

\* Significant at the  $P < 0.05$  level.

<sup>a</sup> Variables: TMB: total microbial biomass, TF: total fungi, AMF: arbuscular mycorrhizal fungi, Sap: saprophytic fungi, TB: total bacteria, Gram +: gram positive bacteria, Gram -: gram negative bacteria, Rhi: Rhizobia, Actin: actinomycetes, Prot: protozoa, F:B: fungi to bacteria ratio, Pr:Py: predator:prey, Mo:po: Mono:polyunsaturated fatty acids, D.I: diversity index, St:Ut: saturated:unsaturated fatty acids, FDA: Fluorescein diacetate hydrolase, BGD:  $\beta$ -glucosidase, BGM:  $\beta$ -glucosaminidase, DHA: dehydrogenase.



Table 3.4. Overall pearson correlation coefficients (r-value) among soil variables for non-treated control at Chariton County Soil Health Farm, Missouri.

Variable <sup>a</sup>	TMB	TF	AMF	Sap	TB	Gram+	Gram -	Rhi	Actin	Prot	F:B	Pr:Py	Mo:po	D. I	St:Ut	FDA	BGD	DHA	
TF	-.27																		
AMF	-.18	.95**																	
Sap	-.29	.99**	.92**																
TB	-.03	.90**	.91**	.88**															
Gram +	-.20	.98**	.98**	.96**	.94**														
Gram -	-.20	.98**	.98**	.96**	.94**	1**													
Rhi	-.28	.76*	.86**	.71*	.65	.82**	.82**												
Actin	.05	.86**	.88**	.84**	.99**	.90**	.90**	.60											
Prot	-.21	.90**	.88**	.89**	.71*	.87**	.87**	.78*	.66										
F:B	-.48	.87**	.81**	.87**	.60	.81**	.81**	.77*	.52	.93**									
Pr:Py	-.25	.72*	.72*	.71*	.46	.67*	.67*	.72*	.39	.94**	.90**								
Mo:po	.19	-.79*	-.85**	-.75*	-.85**	-.81**	-.81**	-.62	-.83**	-.72*	-.60	-.59							
D. I	-.44	.80**	.84**	.78*	.57	.79*	.79*	.88**	.49	.92**	.95**	.93**	-.66						
St:Ut	.44	-.81**	-.84**	-.79*	-.59	-.79*	-.79*	-.81**	-.52	-.88**	-.94**	-.88**	.68*	-.96**					
FDA	.15	.53	.50	.53	.63	.56	.56	.27	.64	.30	.26	.07	-.34	.16	-.23				
BGD	.49	.17	.21	.16	.45	.23	.23	-.11	.52	-.04	-.20	-.25	-.30	-.23	.12	.31			
DHA	.34	.43	.51	.40	.66	.51	.51	.41	.69*	.37	.09	.19	-.57	.17	-.04	.30	.29		
BGM	.45	.53	.48	.54	.58	.51	.51	.21	.58	.61	.33	.49	-.47	.27	-.20	.29	.33	0.68*	

\*\* Significant at the  $P < 0.01$  level.

\* Significant at the  $P < 0.05$  level.

<sup>a</sup>Variables: TMB: total microbial biomass, TF: total fungi, AMF: arbuscular mycorrhiza fungi, Sap: saprophytic fungi, TB: total bacteria, Gram +: gram positive bacteria, Gram -: gram negative bacteria, Rhi: Rhizobia, Actin: actinomycetes, Prot: protozoa, F:B: fungi to bacteria ratio, Pr:Py: predator:prey, Mo:po: Mono:polyunsaturated fatty acids, D. I: diversity index, St:Ut: saturated:unsaturated fatty acids, FDA: fluorescein diacetate hydrolase, BGD:  $\beta$ -glucosidase, BGM:  $\beta$ -glucosaminidase, DHA: dehydrogenase.

Table 3.5. Biomass ( $\text{ng g}^{-1}$ ) of major microbial groups among treatments in October 2014 in the greenhouse study.

Community variable	Treatments			
	Trt4 (150L probiotic $\text{ha}^{-1}$ $\text{yr}^{-1}$ )	Trt3 (120L probiotic $\text{ha}^{-1}$ $\text{yr}^{-1}$ )	Trt2 (90L probiotic $\text{ha}^{-1}$ $\text{yr}^{-1}$ )	Control
	-----ng $\text{g}^{-1}$ -----			
Total biomass	4611 <sup>a</sup>	4643 <sup>a</sup>	4701 <sup>a</sup>	3744 <sup>a</sup>
Total Bacteria	2342 <sup>a</sup>	2137 <sup>ab</sup>	2128 <sup>ab</sup>	1802 <sup>b</sup>
Total Fungi	523.7 <sup>a</sup>	775.6 <sup>a</sup>	710.5 <sup>a</sup>	702.0 <sup>a</sup>
Protozoa	37.99 <sup>b</sup>	66.21 <sup>ab</sup>	78.6 <sup>a</sup>	54.2 <sup>ab</sup>
Saprophytic fungi	385.6 <sup>a</sup>	613.1 <sup>a</sup>	456.1 <sup>a</sup>	448.6 <sup>a</sup>

Different letters within rows denote significant difference at  $p < 0.10$ .

Table 3.6. Biomass (ng g<sup>-1</sup>) of major microbial groups among treatments in December 2014 in the greenhouse study.

Community variable	Treatments			
	Trt4 (150L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Trt3 (120L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Trt2 (90L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Control
	-----ng g <sup>-1</sup> -----			
Total biomass	4369 <sup>ab</sup>	5640 <sup>a</sup>	3862 <sup>b</sup>	3954 <sup>b</sup>
Total Bacteria	2190 <sup>ab</sup>	2738 <sup>a</sup>	1790 <sup>b</sup>	1786 <sup>b</sup>
Total Fungi	350.3 <sup>b</sup>	931.6 <sup>a</sup>	692.3 <sup>ab</sup>	509.4 <sup>ab</sup>
Protozoa	27.05 <sup>b</sup>	69.08 <sup>ab</sup>	82.11 <sup>a</sup>	41.8 <sup>ab</sup>
Saprophytic fungi	214.8 <sup>b</sup>	699.5 <sup>a</sup>	437.8 <sup>ab</sup>	287.8 <sup>b</sup>

Different letters within rows denote significant difference at  $p < 0.10$ .

Table 3.7.  $\beta$ -glucosidase enzyme activity among treatments in August (before probiotic application), October and December 2014 in the greenhouse study.

Sampling time	Treatments			
	Trt4 (150L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Trt3 (120L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Trt2 (90L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Control
	----- $\mu\text{g } p\text{NP g}^{-1} \text{ soil h}^{-1}$ -----			
Aug-2014	5.21	4.91	4.99	5.08
Oct-2014	4.91	4.88	5.10	4.51
Dec-2014	4.30 <sup>b</sup>	5.69 <sup>a</sup>	5.01 <sup>ab</sup>	5.00 <sup>ab</sup>

Different letters within rows denote significant difference at  $p < 0.10$ .

Table 3.8. Fluorescein diacetate hydrolase (FDA) enzyme activity among treatments in August (before probiotic application), October and December 2014 in the greenhouse study.

Sampling time	Treatments			
	Trt4 (150L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Trt3 (120L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Trt2 (90L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Control
	----- µg fluorescein g <sup>-1</sup> soil h <sup>-1</sup> -----			
Aug-2014	4922	4230	4734	4284
Oct-2014	5641	5658	4740	4656
Dec-2014	4158 <sup>d</sup>	6144 <sup>c</sup>	5262 <sup>b</sup>	6732 <sup>a</sup>

Different letters within rows denote significant difference at  $p < 0.10$ .

Table 3.9. Dehydrogenase (DHA) enzyme activity among treatments in August (before probiotic application), October and December 2014 in the greenhouse study.

Sampling time	Treatments			
	Trt4 (150L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Trt3 (120L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Trt2 (90L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Control
	----- µg fluorescein g <sup>-1</sup> soil h <sup>-1</sup> -----			
Aug-2014	5.41	6.40	6.59	6.90
Oct-2014	6.71	7.12	6.51	5.29
Dec-2014	4.84 <sup>c</sup>	13.6 <sup>a</sup>	7.96 <sup>b</sup>	9.97 <sup>b</sup>

Different letters within rows denote significant difference at  $p < 0.10$ .

Table 3.10. Mean values for  $\beta$ -glucosaminidase enzyme activity among treatments in August (before probiotic application), October and December 2014 in the greenhouse study.

Sampling time	Treatments			
	Trt4 (150L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Trt3 (120L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Trt2 (90L probiotic ha <sup>-1</sup> yr <sup>-1</sup> )	Control
	----- $\mu\text{g } p\text{NP g}^{-1} \text{ soil h}^{-1}$ -----			
Aug-2014	1.78	2.12	1.89	2.09
Oct-2014	2.97	2.39	2.40	3.12
Dec-2014	3.25	3.63	3.28	3.23

Different letters within rows denote significant difference at  $p < 0.10$ .

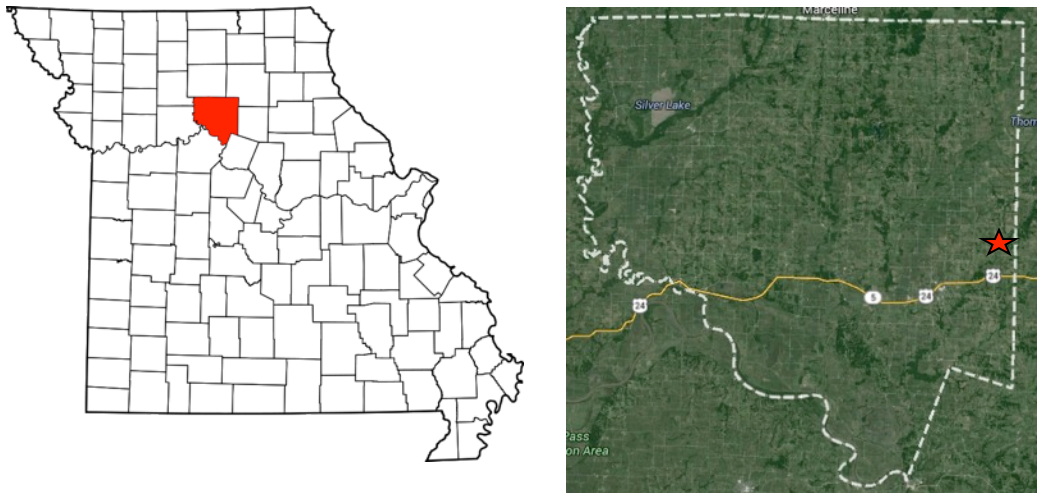


Figure 3.1. Map of Missouri showing the location of Chariton County (L) and Map of Chariton County, star shows the approximate site location (R) of the study site.



Figure 3.2. Photographs showing approximate location of the study site (L) and experimental area (R) at the Chariton cover crop study site.

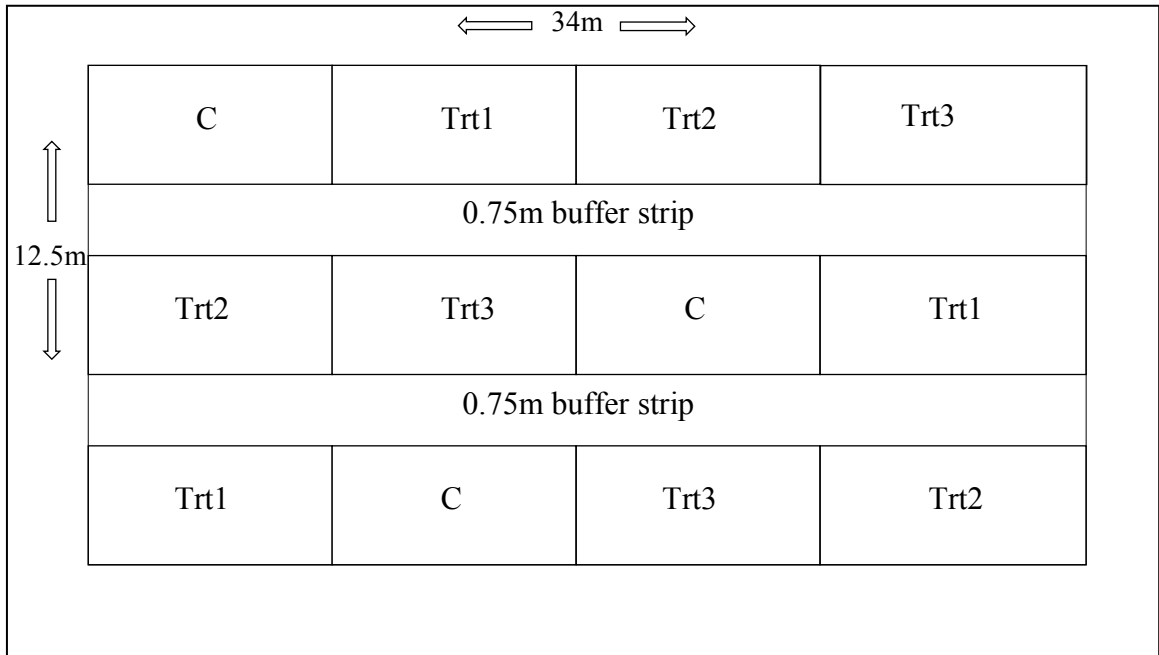


Figure 3.3. Field lay out of the study design at Chariton Cover Crop Study. Letters denote treatments: control; C (1200mL water), treatment 1; Trt1 (72mL Bio-Ag/1128mL water), treatment 2; Trt2 (108mL Bio-Ag/1092mL water), and treatment 3; Trt3 (144mL Bio-Ag/1056mL water).



Figure 3.4. Collecting soil cores using a Giddings soil core sampler for the greenhouse study at Chariton County Soil Health Farm, Missouri.

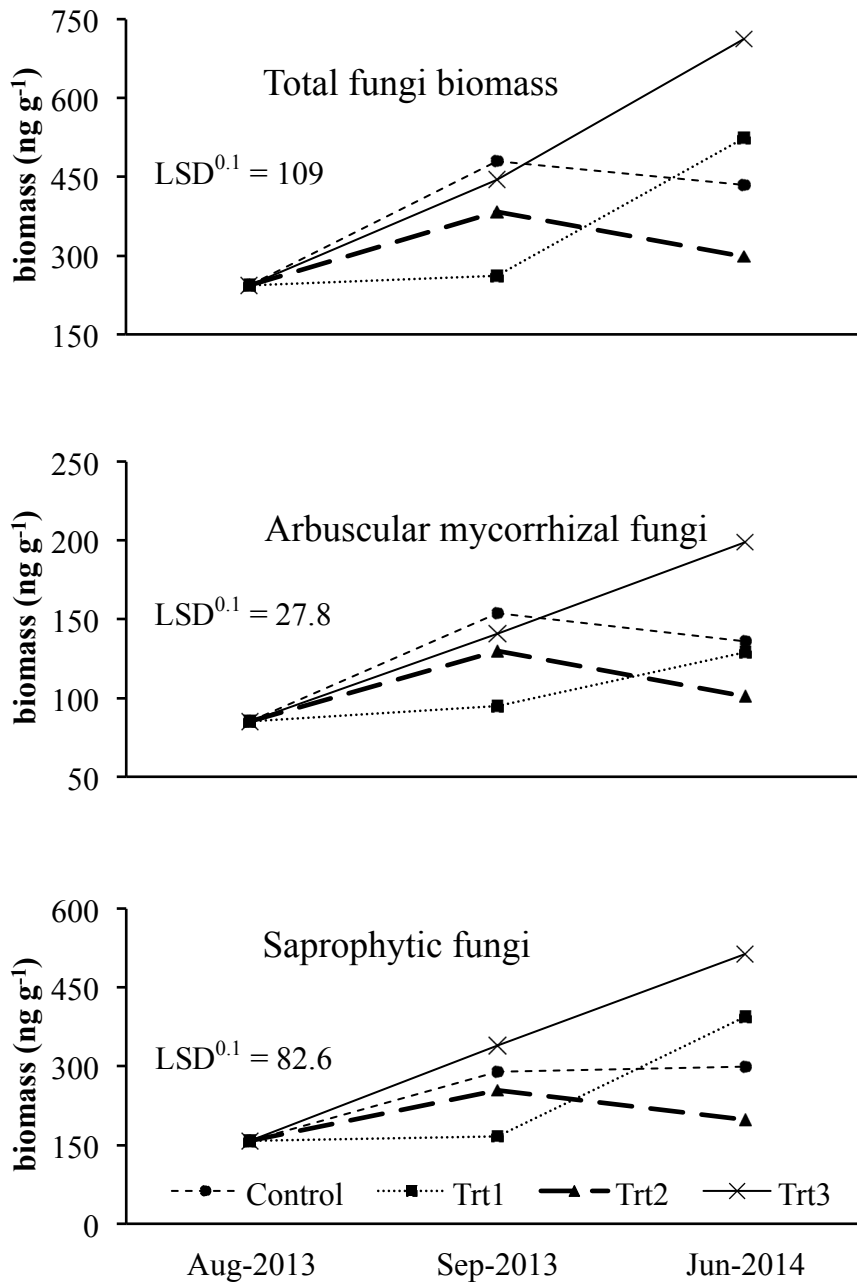


Figure 3.5. Total fungi, arbuscular mycorrhizae fungi (AMF) and saprophytic fungi biomass (ng g<sup>-1</sup>) before the application of probiotic (August 2013), after the first split application of probiotic (September 2013), and after the second split application of probiotic (June 2014) among non-treated control, treatment 1: Trt1 (60L probiotic ha<sup>-1</sup> yr<sup>-1</sup>), treatment 2: Trt2 (90L probiotic ha<sup>-1</sup> yr<sup>-1</sup>), and treatment 3: Trt3 (120L probiotic ha<sup>-1</sup> yr<sup>-1</sup>) at Chariton County Soil Health Farm, Missouri.



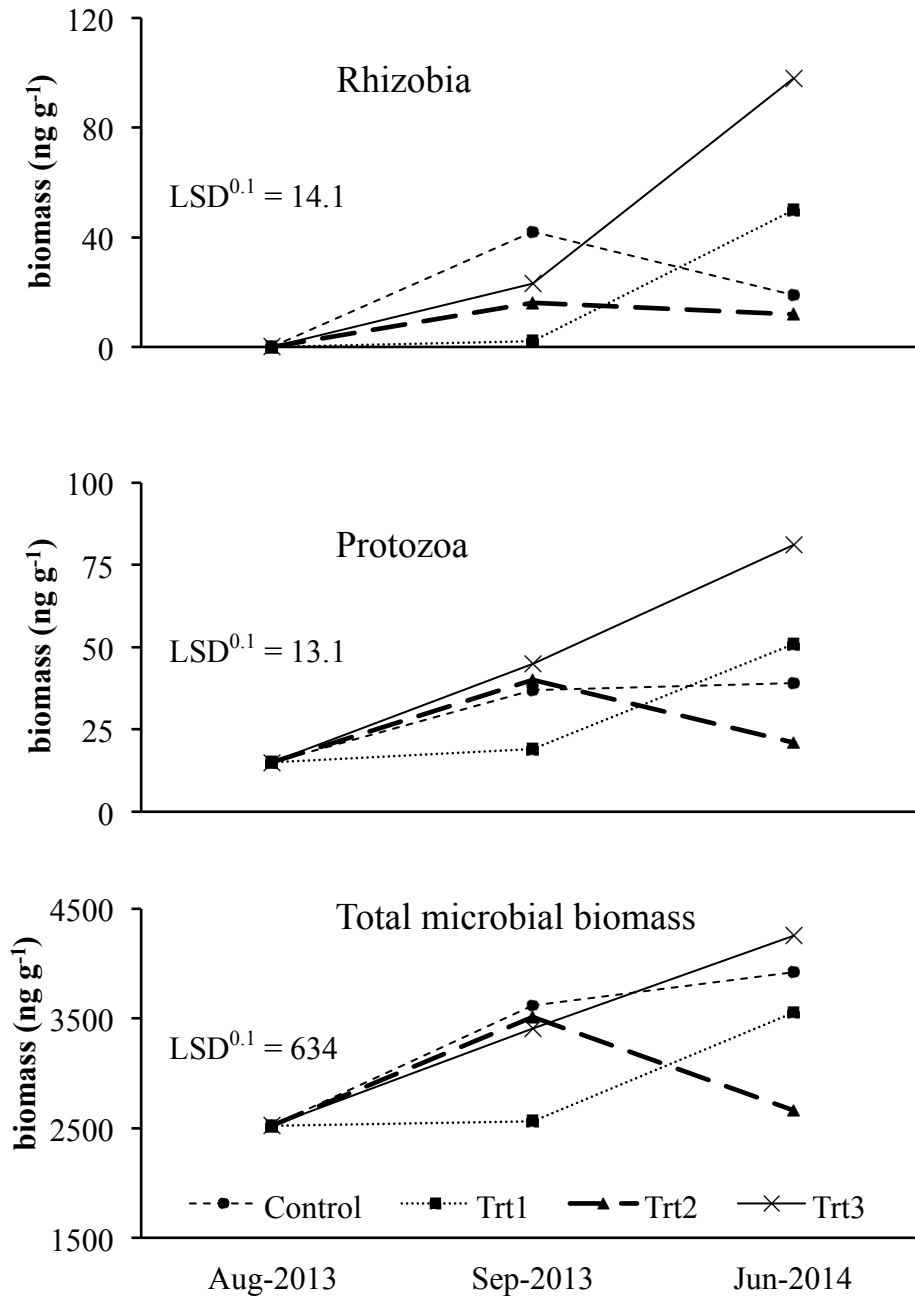


Figure 3.6. Rhizobia, protozoa and total biomass ( $\text{ng g}^{-1}$ ) before the application of probiotic (August 2013), after the first split application of probiotic (September 2013), and after the second split application of probiotic (June 2014) among non-treated control, treatment 1: Trt1 ( $60\text{L probiotic ha}^{-1} \text{ yr}^{-1}$ ), treatment 2: Trt2 ( $90\text{L probiotic ha}^{-1} \text{ yr}^{-1}$ ), and treatment 3: Trt3 ( $120\text{L probiotic ha}^{-1} \text{ yr}^{-1}$ ) at Chariton County Soil Health Farm, Missouri.

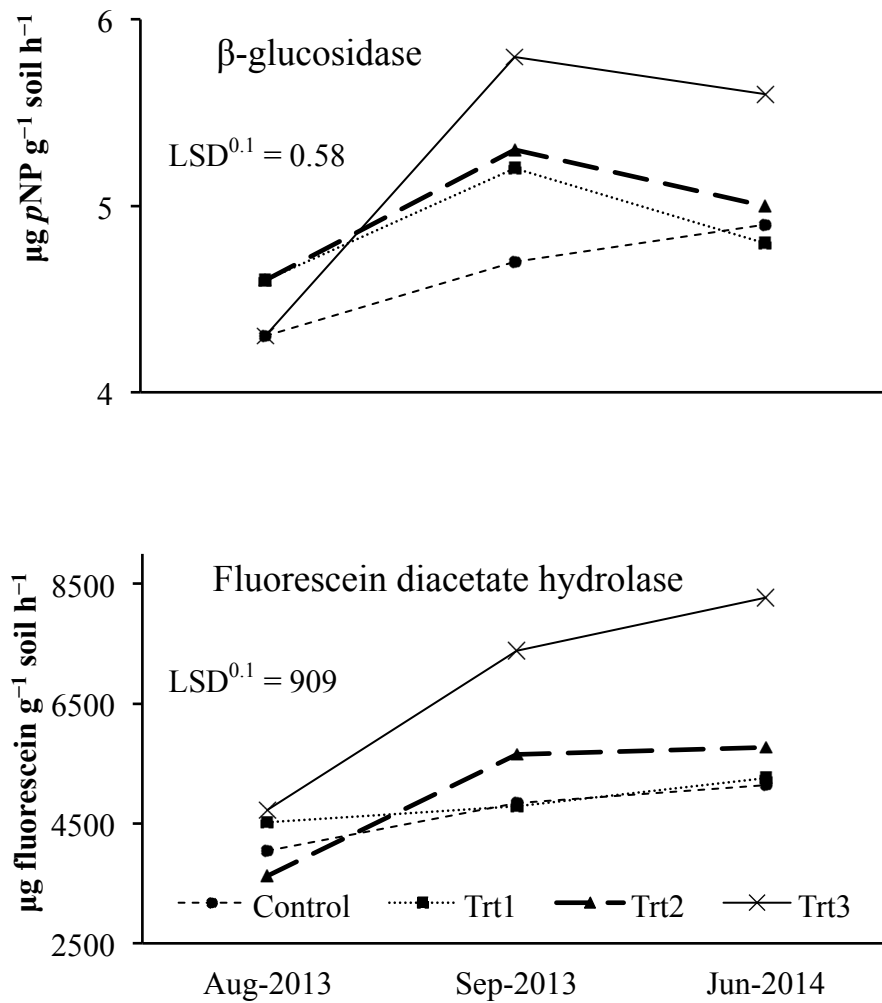


Figure 3.7.  $\beta$ -glucosidase and fluorescein diacetate hydrolase (FDA) activity before the application of probiotic (August 2013), after the first split application of probiotic (September 2013), and after the second split application of probiotic (June 2014) among non-treated control, treatment 1: Trt1 (60L probiotic ha<sup>-1</sup> yr<sup>-1</sup>), treatment 2: Trt2 (90L probiotic ha<sup>-1</sup> yr<sup>-1</sup>), and treatment 3: Trt3 (120L probiotic ha<sup>-1</sup> yr<sup>-1</sup>) at Chariton County Soil Health Farm, Missouri.

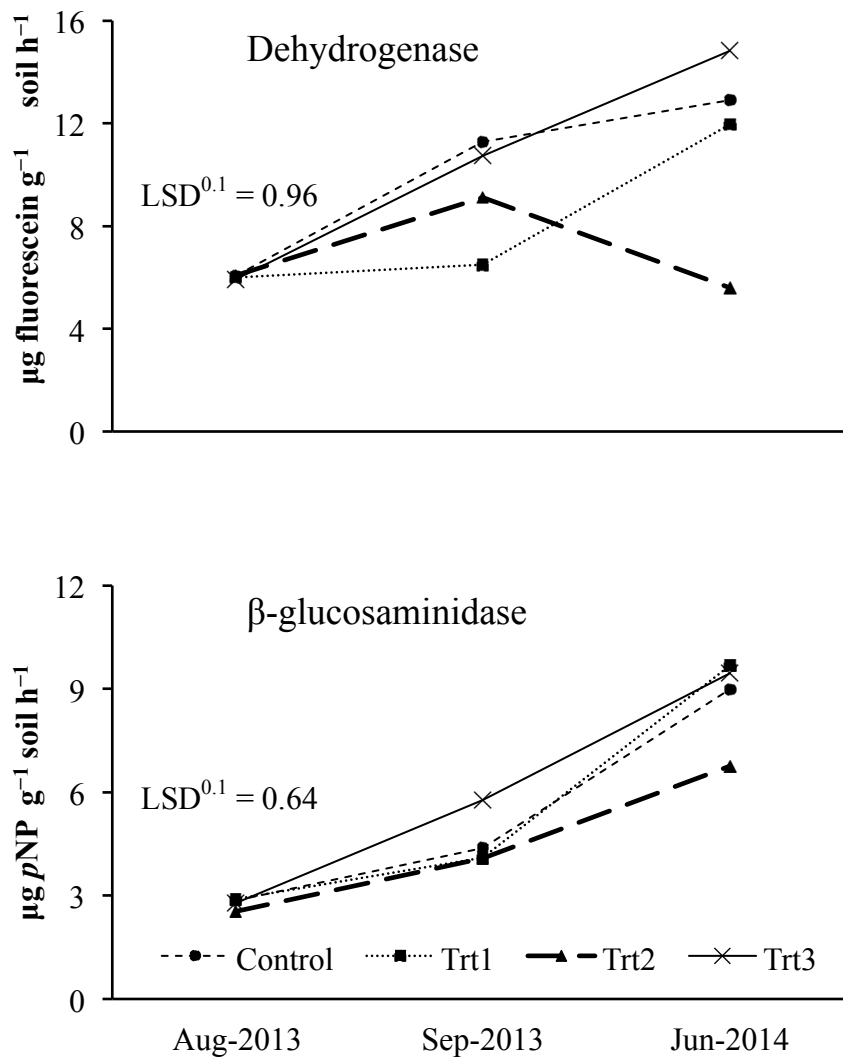


Figure 3.8. Dehydrogenase (DHA) and  $\beta$ -glucosaminidase activity before the application of probiotic (August 2013), after the first split application of probiotic (September 2013), and after the second split application of probiotic (June 2014) among non-treated control, treatment 1: Trt1 (60L probiotic  $\text{ha}^{-1} \text{ yr}^{-1}$ ), treatment 2: Trt2 (90L probiotic  $\text{ha}^{-1} \text{ yr}^{-1}$ ), and treatment 3: Trt3 (120L probiotic  $\text{ha}^{-1} \text{ yr}^{-1}$ ) at Chariton County Soil Health Farm, Missouri.

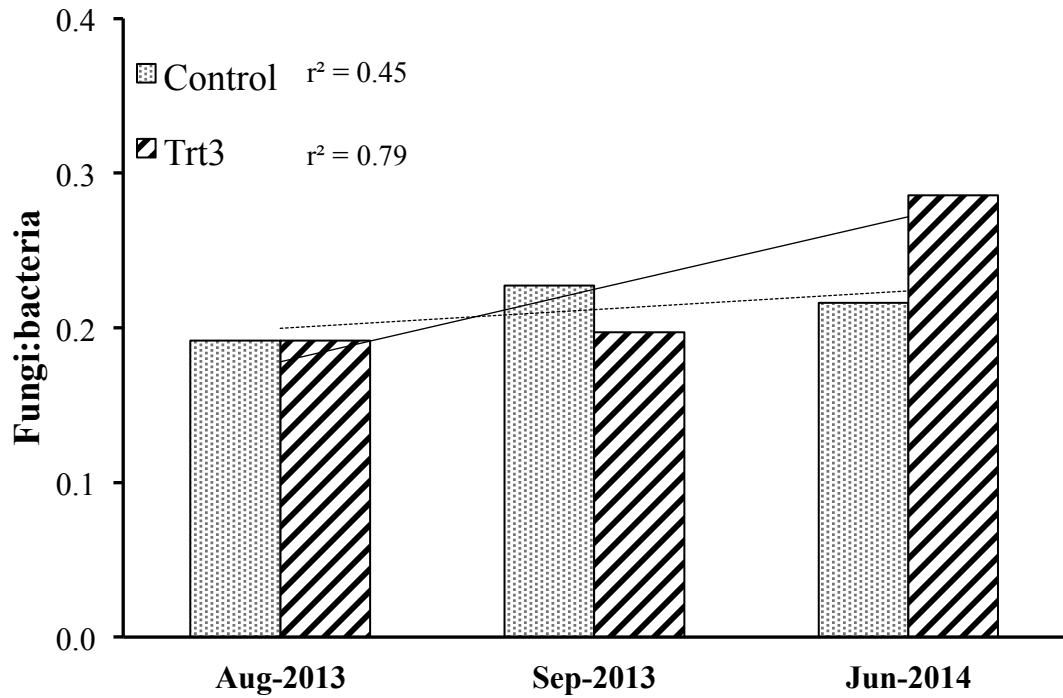


Figure 3.9. Fungi to bacteria ratio before the application of probiotic (August 2013), after the first split application of probiotic (September 2013), and after the second split application of probiotic (June 2014) between non-treated control and treatment 3: Trt3 (120L probiotic ha<sup>-1</sup> yr<sup>-1</sup>) at Chariton County Soil Health Farm, Missouri.

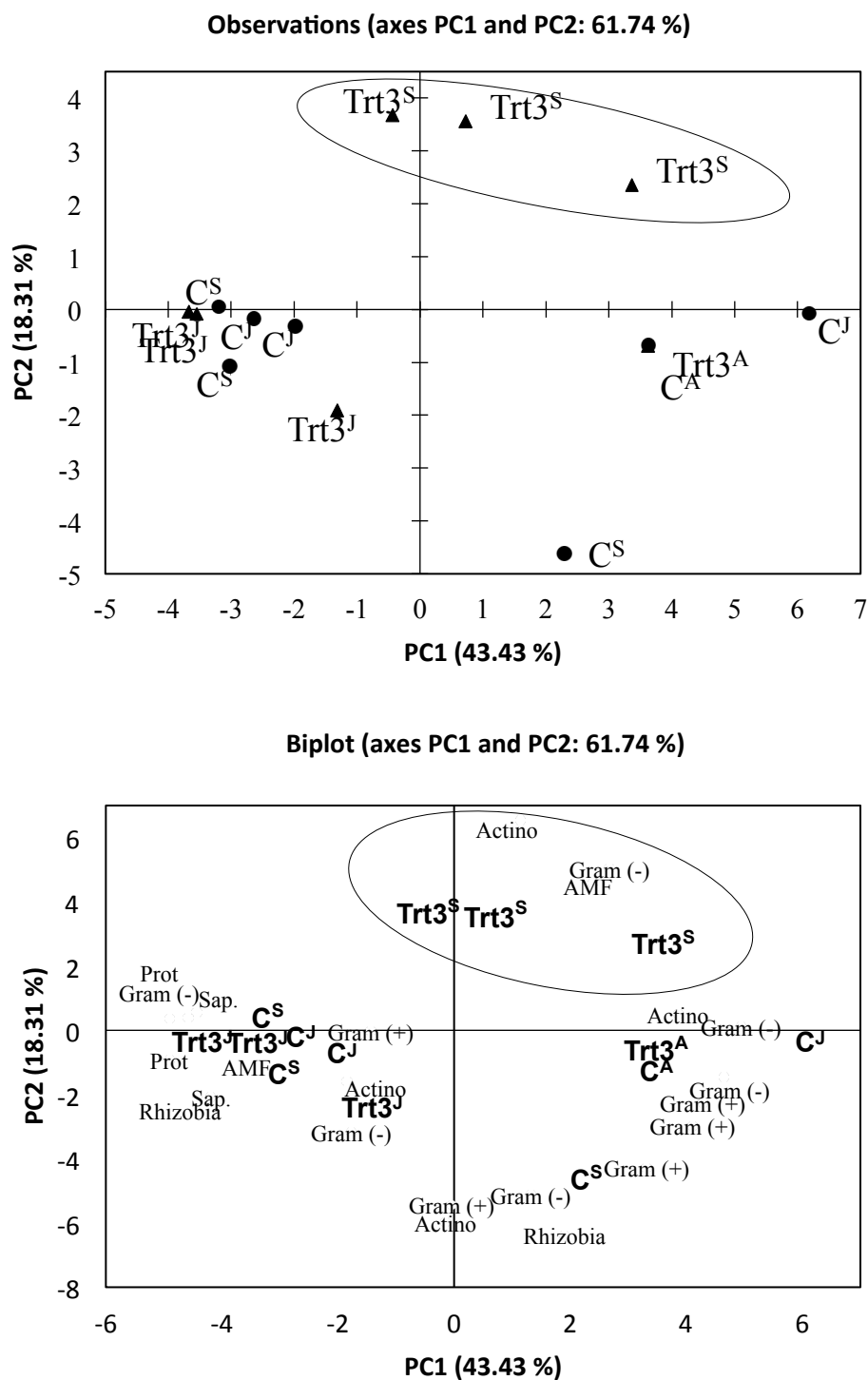
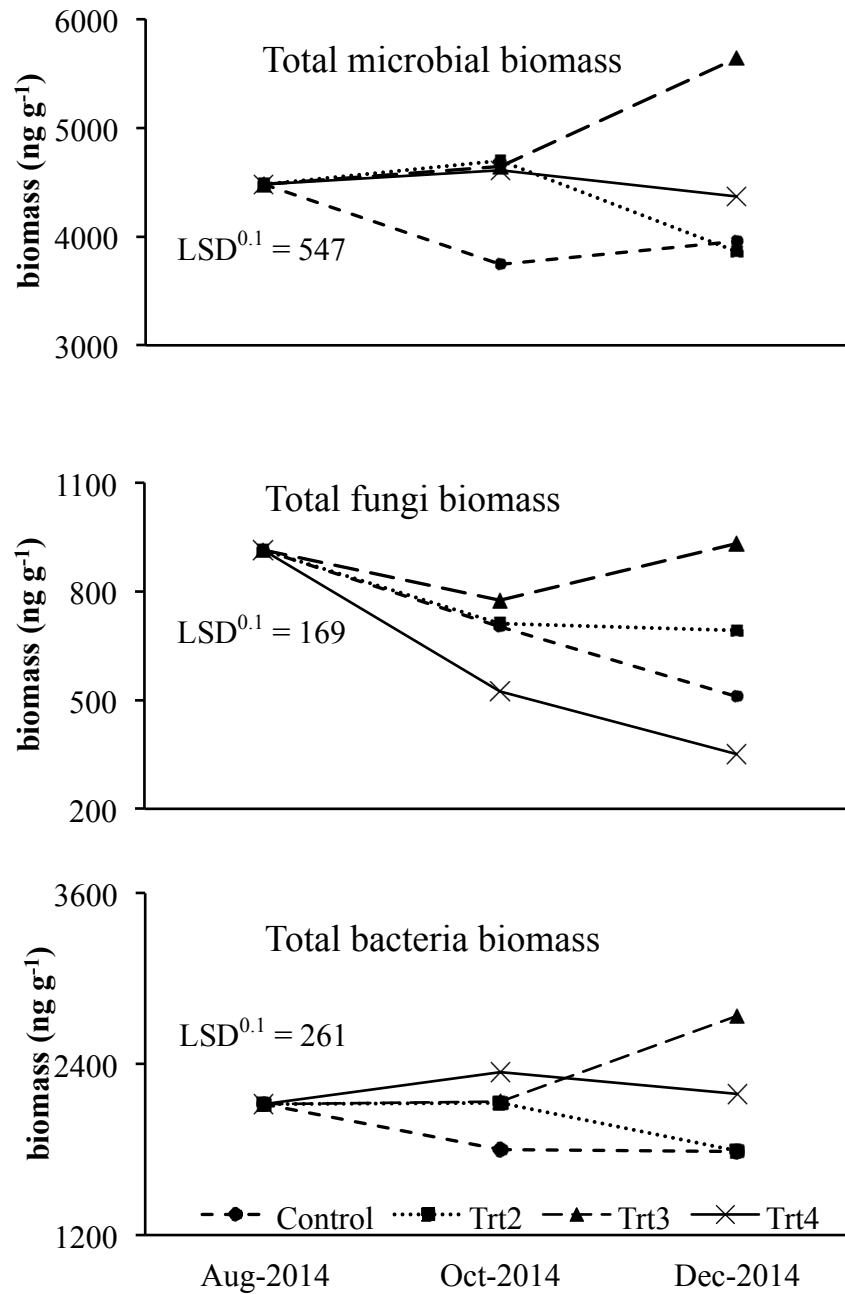


Figure 3.10. Principal component analysis of treatment 3: Trt3 (120L probiotic ha<sup>-1</sup> yr<sup>-1</sup>) and non-treated control: C at Chariton County Soil Health Farm, Missouri. Superscripts A, S and J denote sampling date Aug-2013, Sep-2013, and Jun-2014 respectively. Actino, Sap., Gram (+), Gram (-), AMF and Prot stands for actinomycetes, saprophytic fungi, gram-positive bacteria, gram-negative bacteria, arbuscular mycorrhizae fungi and protozoa respectively.



Figure

3.11. Total microbial, fungi and bacteria biomass ( $\text{ng g}^{-1}$ ) before the application of probiotic (August 2014), after the first split application of probiotic (October 2014), and after the second split application of probiotic (December 2014) among non-treated control, treatment 2: Trt2 (90L probiotic  $\text{ha}^{-1} \text{yr}^{-1}$ ), treatment 3: Trt3 (120L probiotic  $\text{ha}^{-1} \text{yr}^{-1}$ ), and treatment 4: Trt4 (150L probiotic  $\text{ha}^{-1} \text{yr}^{-1}$ ) in the greenhouse study.

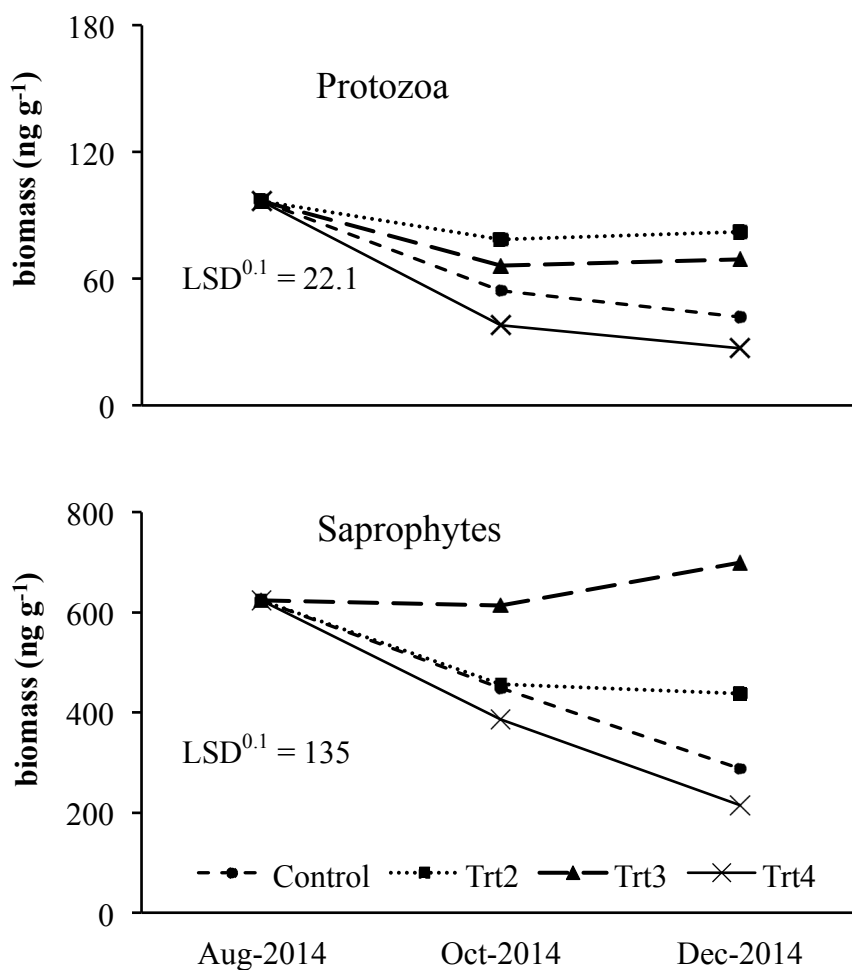


Figure 3.12. Protozoa and saprophytic fungi biomass (ng g<sup>-1</sup>) before the application of probiotic (August 2014), after the first split application of probiotic (October 2014), and after the second split application of probiotic (December 2014) among non-treated control, treatment 2: Trt2 (90L probiotic ha<sup>-1</sup> yr<sup>-1</sup>), treatment 3: Trt3 (120L probiotic ha<sup>-1</sup> yr<sup>-1</sup>), and treatment 4: Trt4 (150L probiotic ha<sup>-1</sup> yr<sup>-1</sup>) in the greenhouse study.

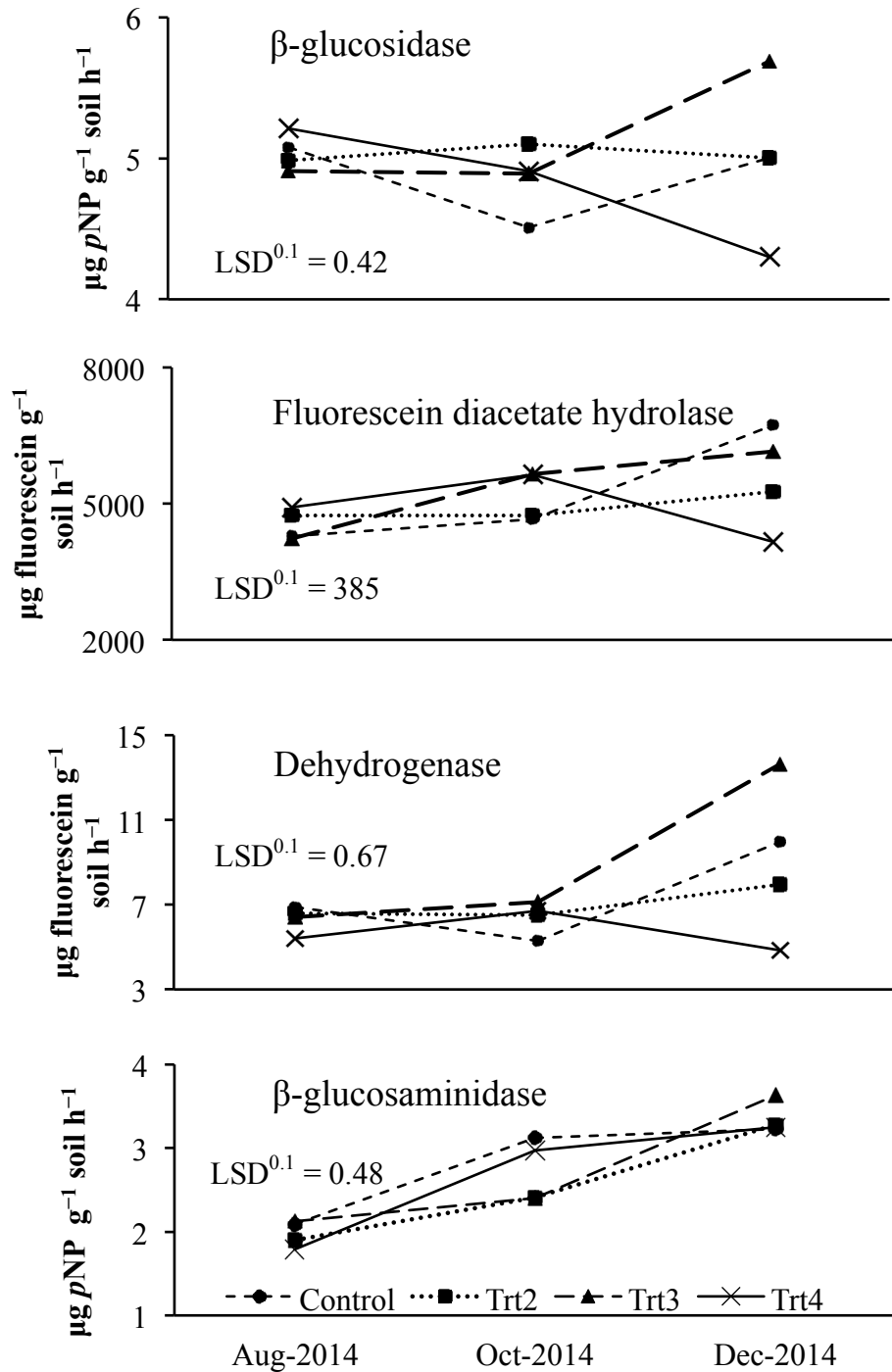


Figure 3.13.  $\beta$ -glucosidase, fluorescein diacetate hydrolase (FDA), dehydrogenase (DHA) and  $\beta$ -glucosaminidase activity before the application of probiotic (August 2014), after the first split application of probiotic (October 2014), and after the second split application of probiotic (December 2014) among non-treated control, treatment 2: Trt2 (90L probiotic  $\text{ha}^{-1} \text{ yr}^{-1}$ ), treatment 3: Trt3 (120L probiotic  $\text{ha}^{-1} \text{ yr}^{-1}$ ), and treatment 4: Trt4 (150L probiotic  $\text{ha}^{-1} \text{ yr}^{-1}$ ) in the greenhouse study.



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## CHAPTER 4

# PROBIOTIC INFLUENCE ON HAIRY VETCH ROOT – MICROBIAL RELATIONSHIPS AND PRECURSOR- INDEPENDENT AUXIN PRODUCTION IN SOILS

### 4.1. Abstract

Probiotics are believed to improve plant growth, root development and production of plant growth promoting substances. The objective of this study was to quantify probiotic effects on hairy vetch roots and precursor independent auxin production in soils. Undisturbed soil cores (2356cm<sup>3</sup>) were collected using PVC tubes in July 2014 from NRCS soil health farm, Chariton County, Missouri to conduct a greenhouse study with control and treatment 3 (Trt3; 120L ha<sup>-1</sup> yr<sup>-1</sup> of Bio-Ag probiotics) treatments on hairy vetch (*Vicia villosa* Roth.). Soils in the study site are Armstrong loam (fine, smectitic, mesic Aquertic Hapludalfs). Two equal split applications of probiotics were applied in August, and October, 2014. Soil samples were collected from rhizosphere in August and October, 2014 with three replicates. Soils were cultured on selective medium to assess the fluorescent pseudomonad community. Fluorescent pseudomonads were significantly higher in Trt3 than control after the second probiotic application. Auxin production in soil samples was determined by High-performance liquid chromatography-Mass spectrometry (HPLC-MS/MS). Probiotics showed no effect on the precursor-independent production of auxin in soil samples. Two plant root samples were also collected after 7 days of first probiotic application for scanning electron microscopy

(SEM) observations. Images of SEM revealed more root hair growth ( $p < 0.05$ ) and microbial colonization on hairy vetch roots treated with probiotic compared to control.

Results imply that probiotic had positive effect on root hair growth of hairy vetch and Fluorescent pseudomonads population. However, it did not have any effect on precursor-independent production of auxins.

#### **4.2. Introduction**

The term plant growth regulators (PGRs) is used to define biologically active substances that regulate plant growth and influence physiological processes of plants at very low concentrations (Frankenberger and Arshad, 1995). Phytohormones are PGRs that are produced endogenously by plants. However, they can be synthesized by microorganisms if the required substrate is available. Therefore, under certain conditions, plants may not synthesize sufficient endogenous phytohormones for their optimal growth and development and depends on microbial production (Frankenberger and Arshad, 1995). Some of the well-known phytohormones are auxins, gibberellins, cytokinins, ethylene, kinetin and abscisic acid.

Barea et al. (1976) found that 86 and 90% of 50 bacterial strains isolated from rhizosphere of various crops produced auxins and kinetin-like substances, respectively. Auxin is one of the important phytohormones that play a major role in plant growth and development. The chemical structure of one type of auxin is indole-3-acetic acid (IAA;  $C_{10}H_9NO_2$ ) which influences cell enlargement, flowering, fruit growth, protein synthesis, root initiation, and fruit ripening (Frankenberger and Arshad, 1995). More than 80% of the bacteria isolated from the rhizosphere have the capability to synthesize IAA (Khalid

et al., 2004). Reis Júnior et al. (2004), have observed *Azospirillum brasilense* associated with many plants, including maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and oats (*Avena sativa* L.). Glick et al. (1997) have observed a positive effect of pseudomonad strains on root and shoot elongation in canola (*Brassica napus* L.), tomato (*Solanum lycopersicum* L.) and lettuce (*Lactuca sativa* L.). Phosphate solubilizing *Bacillus* spp. stimulates plant growth through P nutrition of wheat (Whitelaw et al., 1997), and increase the uptake of N, P, K and Fe in rice (Biswas et al., 2000). It has been observed that the application of PSMs and PGPRs together can reduce P application by 50% without any significant reduction in grain yield in corn (Yazdani et al., 2009). PSBs also enhance vegetative growth and fruit quality and reduce P pollution of environment (Attia et al., 2009).

When the substrate is available, rhizosphere microorganisms are more active in producing auxin than those from root-free soil (Strzelczyk and Pokojaska-Burdziej, 1984). Tryptophan (TRP) is a precursor of auxin production by plants and microorganisms. Conversion of TRP to IAA occurs through many routes including deamination, decarboxylation and hydrolysis. Microorganisms have been found to produce IAA if TRP or tryptamine (TAM; a metabolite of TRP) is used as a precursor (Frankenberger and Arshad, 1995).

Armada et al. (2014) showed that some bacteria produce small amounts of auxin-like substances in the absence of precursors under natural conditions. Many microorganisms produce IAA or auxin-like substances without addition of precursors including *Arthrobacter* spp. (Strzelczyk and Pokojaska-Burdziej, 1984), *Bacillus* spp. (Ali et al., 2008), and *Rhizobium* spp. (Ernstsen et al., 1987). *Azotobacter* spp. also produce



IAA in the absence of TRP (Müller et al., 1989). However, addition of TRP stimulates IAA production (Zhang et al., 2008). Fallik et al. (1989) found that *Azospirillum brasilense* is capable of producing large amounts of IAA in the absence of TRP. Similar results were obtained by Horemans et al. (1986). Interestingly, Müller et al. (1989) found that *Azospirillum lipoferum* produced more IAA in the absence of TRP than in the presence of TRP. Mycorrhizal fungi also produce small amounts of IAA in the absence of TRP (Ludwig-Müller and Güther, 2007).

Previously, many research studies focused on auxin production in presence of precursors since detectable levels of IAA were not found in the absence of TRP, (Sarwar et al., 1992). Currently available technologies allow determination of these compounds at the parts per billion (ppb) range in soil and plant samples. Pan et al. (2010) described a very precise method using high performance liquid-chromatography (HPLC)-mass spectrometry (MS/MS) for a wide range of phytohormones in plant samples. Frankenburger and Brunner (1983) have described methods for detection of auxin production in soil samples using HPLC-MS/MS method. However, their study was conducted with addition of TRP in soils as a supplemental substrate. Therefore, the range detected was more than 100 parts per million (ppm). No other significant study has been conducted for soil samples. Our method of analysis is similar to that of Pan et al. (2010), however it has been customized since soil samples for the current study were prepared differently. We hypothesize that our protocol for HPLC-MS/MS could allow us to detect analysis for three different auxin compounds produced in soil samples in concentrations of up to 25ppb.

The only natural source of TRP for microorganisms is plant root exudates (Frankenberger and Arshad, 1995). However, very few studies have been conducted on the conversion of natural TRP to IAA through microbial activities since all plants may not release adequate amounts of TRP (Martens and Frankenberger, 1993). Additions of TRP to soils enhances microbial production of IAA and positive effects are shown on the growth of plants (Frankenberger and Arshad, 1995). Although, high concentrations of auxins inhibit root growth (Sarwar and Kremer, 1995), continuous microbially-produced auxins often have beneficial effects on plant growth (Frankenberger and Arshad, 1995). The overall objective of this study was to quantify the effects of probiotics on microbial production of three different auxins in soils, by conversion of natural substrates and study the effect of probiotic on hairy vetch root development.

### **4.3. Materials and Methods**

#### **4.3.1. Treatments and sampling**

Undisturbed soil cores (10cm diam. 30cm long; 2356cm<sup>3</sup>) were collected from the NRCS Soil Health Farm, Chariton country, Missouri in PVC tubes in July 2014. PVC sections were placed on the soil surface and pushed into the soils using Gator mounted Giddings probe. Once taken, soil cores were sealed from both sides with plastic caps. Duct tape was used to secure soils inside the tube and brought for the greenhouse study.

Study included two treatments: control and treatment 3 (Trt3; 120L ha<sup>-1</sup> yr<sup>-1</sup> of SCD Bio-Ag Probiotics). Probiotics used in this study (SCD Bio-Ag) contained following microbial species: *Bacillus subtilis*, *bifidobacterium animalis*, *b. bifidum*, *b. longum*, *Lactobacillus acidophilus*, *L. bulgaricus*, *L. casei*, *L. plantarum*, *L. fermentum*,

*Lactococcus lactis*, *Rhodopseudomonas sphaeroides*, *R. Palustris*, *Saccharomyces cerevisiae* and *Streptococcus thermophiles* with concentrations of  $>3.0 \times 10^5$  cfu (colony-forming unit)  $\text{ml}^{-1}$  lactic acid bacteria and  $<1.0 \times 10^6$  cfu  $\text{ml}^{-1}$  yeast (SCD Probiotics, Kansas City, MO).

Hairy vetch cover crop was seeded on July 07, 2014 with 4-6 seeds in each soil core. Two equal amounts of probiotics were applied in August, and October, 2014 respectively. Triplicate soil samples were collected from rhizosphere after removing the plants carefully from each soil core in August, and October, 2014 seven days after probiotic application. Two plant root samples were also collected seven days after probiotic application from control and Trt3 treatments. Samples were placed in labeled zip lock bags and stored at 4°C before the analysis.

#### **4.3.2. Fluorescent pseudomonad population density**

Fluorescent pseudomonads were selectively cultured on King's B agar medium from soil suspensions (Pepper and Gerba, 2005). One gram moist soil was suspended in 9ml 0.01M  $\text{MgSO}_4$  and serial ten-fold dilutions of 0.001, 0.0001, and 0.00001 were prepared (Appendix B). A glass spreader was dipped in ethanol and flame-sterilized. A 0.1ml suspension aliquot was transferred to spread plate and uniformly distributed on plate surface area using brief touch of glass spreader. Spread plates were prepared in duplicate. Spread plates were labeled and incubated for two days. Gravimetric water content of soil samples was determined to quantify soil samples on dry weight basis. One-way ANOVA was performed on the raw numbers obtained from countable dilutions (0.0001 and 0.00001) for October and December 2014 separately.

### **4.3.3. Scanning electron microscopy (SEM)**

FEI Quanta 600 SEM available at Electron Microscopy Core Facility, University of Missouri-Columbia was used to study root colonization by rhizobacteria on hairy vetch cover crop. The equipment was operated on 5 and 10kV voltages with 12mm of working distance. Roots were obtained after 7 days of inoculation of probiotics and cut into 1.5mm thick section. Root samples were prepared using critical point drying (CPD) method. High and low vacuum (variable pressure 53Pa) was used during operation of SEM to decrease the charging issue. Root hairs were quantified by visual observations of four images for each samples from different angles and mean was calculated followed by one-way ANOVA analysis (Table 4.2).

### **4.3.4. High-performance liquid chromatography-Mass spectrometry (HPLC-MS/MS)**

#### **4.3.4.1 Sample preparation**

Two-gram soil samples were extracted with 10ml 9:1 methanol solution (90% methanol: 10%water with 1% formic acid) to analyze for IAA, indole-3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA). Samples were sonicated for 1hr and then vacuum filtered through Whatman filter paper no. 1. Samples were further rinsed with 10ml methanol solution during filtration to pass the analytes into the filtrates efficiently. Filtrates were transferred to the separatory funnel and acidified with 3ml of 1N HCl followed by dilution with 15ml of 10% NaCl. Then liquid-liquid extraction was performed two times with 10ml of dichloromethane ( $\text{CH}_2\text{Cl}_2$ ). Analytes were evaporated using Nitrogen evaporator to almost a dried stage. Finally, analytes were resuspended

with 2ml of acetonitrile (C<sub>2</sub>H<sub>3</sub>N), vortexed, and transferred to vials using syringe and anotop filter (0.2µm). Three blanks and three spike samples (500ppb) were prepared to observe recovery efficiency.

Stock solutions (100ppm) of HPLC grade indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) (Sigma Aldrich, St. Louis, MO) were prepared followed by 10, 5, 1, 0.5, 0.25, 0.10, 0.05 and 0.025ppm serial dilutions in acetonitrile (CH<sub>3</sub>CN) for standards preparation to obtain calibration curve. The first dilution (10ppm) was injected separately for three chemicals followed by mixing remaining dilutions of all chemicals and injected together as a mixture.

#### **4.3.4.2. Instrumentation**

The concentrations of IAA, IBA, and NAA were determined using Waters Alliance 2695 High Performance Liquid Chromatography system coupled with Waters Acquity TQ triple quadrupole mass spectrometer (HPLC-MS/MS). The PHCs were separated by a Phenomenex (Torrance, CA) Kinetex C18 (100mm x 4.6 mm; 2.6 µm particle size) reverse-phase column. The mobile phase consisted of 10 mM ammonium acetate and 0.1% formic acid in water (A) and 100% acetonitrile (B). The gradient conditions were 0–0.5 min, 2% B; 0.5–7 min, 2–80% B; 7.0–9.0 min, 80–98% B; 9.0–10.0 min, 2% B; 10.0–15.0 min, 2% B at a flow rate of 0.5 ml/min. The MS/MS system was operated using electrospray ionization (EI) in the negative ion mode with capillary voltage of 1.5 kV (ES<sup>-</sup>). The ionization source was programmed at 150°C and the desolvation temperature was programmed at 450°C. The MS/MS system was operated in the multi-reaction monitoring (MRM) modes and the collision energy was 12V, 18V and

30V for IAA, IBA and NAA, respectively. The molecular parent ions were screened and the product ions used for the quantifications were determined from the spectra obtained from injecting 30mL of a standard solution containing 1000mg L<sup>-1</sup> of the analytical standards. Analytical data were processed using Waters Empower software (Waters, CA, USA). The detailed selection of the ions, retention times and ionization parameters are described as in Table 4.3.

#### **4.4. Results and Discussion**

##### **4.4.1. Fluorescent pseudomonads**

Mean fluorescent pseudomonad numbers (cfu's) in rhizosphere soil were higher in Trt3 (120L ha<sup>-1</sup> yr<sup>-1</sup>) sampled after the first application (Oct-2014) but did not differ significantly from the control. Fluorescent pseudomonads were significantly higher ( $p < 0.05$ ) in Trt3 sampled after the second application (Dec-2014) than the control (Fig. 4.1 and Table 4.1). Tyc et al. (2015) found that cell numbers of *P. fluorescens* were significantly reduced when confronted with *Bacillus* sp. in a study conducted in sand microcosms, which contradicts our results. However, our results suggest that the fluorescent pseudomonad population increased when soil was inoculated with the microorganisms present in the probiotic, which also contained *Bacillus* spp. Tyc et al. (2015) observed the confrontation of only one specific *P. fluorescens* strain (Pf0-1) with *Bacillus* spp. while our experiment considered all fluorescent *Pseudomonas* spp. present in the field-collected soil samples. Future studies can focus on isolation and identification of fluorescent pseudomonad strains from soils to confirm the increased composition of *Pseudomonas* spp. in response to addition of probiotic.

*Pseudomonas fluorescens* is widely used as a biocontrol including functioning as an agent against various phytopathogens in radish (*Raphanus sativus* L.), tomato (*Solanum lycopersicum* L.), wheat (*Triticum aestivum* L.), tobacco (*Nicotiana tabacum* L.) and arabidopsis (*Arabidopsis thaliana* L.) (Duijff et al., 1997; Leeman et al., 1995; Pieterse et al., 2000). However, threshold populations of *P. fluorescens* for significant suppression varies with different plants and depends on specific *P. fluorescens* strains (Raaijmakers and Weller, 1998). Moreover, plant growth by various mechanisms mediated by pseudomonads such as production of antibiotics, induced systemic resistance, nutrient uptake from soil, and producing plant-growth-promoting substances (i.e., auxins) (De Weger et al., 1986; Ryu et al., 2005; Spaepen et al., 2007).

#### **4.4.2. Root structure and microbial colonization**

Microbial density appeared greater on the probiotic treated roots compared with the non-inoculated control (Fig. 4.3). Moreover, greater root hair development was observed ( $p < 0.001$ ) under probiotic treated roots as compared to control (Fig. 4.2 and Table 4.2). Only the root hairs, which were positioning to the outside, were taken into the consideration and quantified. Standard error was higher in Trt3 (2.56) than control (2.38) with negligible difference. However, total standard error (4.33) was highest due to limited number of replicate images and samples. Biofilm-like matrices were observed in the probiotic-treated roots and control (Fig. 4.3), but no visual differences were found between treatments. However, overall microbial density was higher on probiotic-treated roots than controls and more bacillus-like density was observed in probiotic treated root (Fig 4.3). *Bacillus* sp. have been reported to promote plant growth in many crops

including soybean, tomato, and wheat, and also known to produce auxins (Deepa et al., 2010; Bai et al., 2003; Kokalis-Burelle et al., 2002; Kloepper et al., 2004; Zhang et al., 2008). It is also known to repress soil-borne pathogens and work as biocontrol agents in field and greenhouse studies (Stabb et al., 1994; Kloepper et al., 2004).

Some blurriness was found in control sample images of SEM. Due to that reason, high and low vacuum (variable pressure 53 Pa) was used in SEM to decrease the charging issue in control sample. Charging is the result of electrons from the electron beam building up in a nonconductive sample. Extra electrons then jump from the sample unpredictably causing lines and blurriness on the image. With variable pressure, charging is decreased by more release of hydroxyl ions.

#### **4.4.1. Auxin content in soil samples**

No auxin (IAA, IBA and NAA) was detectable (detection limit of 25ppb for each auxins compound) in any of the soil samples. However, the method developed was operational as standards, blanks and spiked samples were also analyzed using same protocol to test the developed method (Table 4.4). Recovery efficiency for NAA was 87%, which is very precise. Variance among spikes for IAA and IBA recovery rate could be due to limited number of spikes and precision of sample preparation. In future, more numbers of spikes could be included to further justify the recovery rate for IAA and IBA since this protocol can be used to analyze auxin-like compounds. Furthermore, extracts can be concentrated to detect auxin up to 1-2 ppb. However, concentrations of auxins less than 1 ppm have been observed and are usually considered insignificant (Mohite, 2013). Asghar et al. (2002) observed that root and shoot length of indian mustard



(*Brassica juncea* L.) was increased when IAA was detected at more than 5ppm in soils. No effect on root and shoot of indian mustard was found when IAA concentration was less than 1ppm. Tien et al. (1979) found that roots of pearl millet (*Pennisetum americanum* L.) were increased in length and density when the medium was inoculated with tryptophan and specific isolate, with IAA concentration of more than 2ppm. However, all of the studies mentioned above were conducted after rhizobacterial isolation from soils and isolates were inoculated with an external source of TRP. Because our soils were not amended with an external substrate (i.e., TRP) for IAA synthesis, this may explain why no IAA was detected by our analytical procedure. Moreover, such low concentrations of auxins would be insignificant for the plant growth and development. Therefore, future experiments may be conducted using applied external substrate such as TRP.

#### **4.5. Conclusion**

The results of the experiment suggest that probiotics promote bacterial populations on roots indicated by biofilm-like matrices visible on the probiotic-treated roots. More root hair growth was observed in probiotic-treated roots of hairy vetch. Furthermore, similar experiments can be conducted on other crops (wheat, corn, soybean, cover crops). In addition, long-term effects of probiotics on roots can be observed by visualizing the sample inoculated for more than 7 days. Actual root growth data (root length, area, density) can also be collected to further quantify the effects of probiotics.

Fluorescent pseudomonad population was enhanced with probiotic application in the rhizosphere, which is one of the main driver of auxin and many other phytohormone

production in soil. Results imply the indirect positive effects of probiotic on auxin and other phytohormones can be confirmed in the future by conducting more research on probiotic and its role in enhancing plant growth substances such as phytohormones.

We could not detect precursor-independent auxin content in soil less than 25ppb for any of the auxins compound (IAA, NAA, IBA). One of the reasons could be absence of precursor. For instance, TRP in the rhizosphere is one of the main precursors for IAA production in the soils. Results suggest that probiotic does not have any effect on auxin content in soils in the absence of external source of substrate under the conditions of this study.

#### 4.6. Tables and Figures

Table 4.1. Colony forming units count of fluorescent pseudomonads on spread plate.

Treatments	Dilution factor
	0.0001
Oct-2014	Colony forming units (cfu) g <sup>-1</sup> dry soil
Control	9.63x10 <sup>5</sup>
Trt3 (120L ha <sup>-1</sup> yr <sup>-1</sup> )	1.27x10 <sup>6</sup>
Dec-2014	
Control	9.41x10 <sup>5</sup>
Trt3 (120L ha <sup>-1</sup> yr <sup>-1</sup> )	3.04x10 <sup>6</sup>

Table 4.2. Number of root hairs quantified on 36-day old hairy vetch roots by visual observation of scanning electron microscopy (SEM) images on probiotic treated and non-treated control sample.

Replicate image	Number of hairs		Area
	Control	Trt3 (120L ha <sup>-1</sup> yr <sup>-1</sup> )	
1	8	23	1mm <sup>2</sup>
2	10	32	1mm <sup>2</sup>
3	2	21	1mm <sup>2</sup>
4	0	29	1mm <sup>2</sup>
Mean	<b>5</b>	<b>26</b>	1mm <sup>2</sup>
Std. deviation	4.76	5.12	
Std. error	2.38	2.56	

Table 4.3. Selected conditions retention times for deprotonated phytohormones.

	Retention Times (min)	Molecular Ion (M-H) <sup>-</sup>	Product Ion	Cone Energy (V)	Collision Energy (V)
IAA	7.7	174	130	25	12
IBA	8.6	202	158	40	18
NAA	8.8	185	141	40	30

Table 4.4. Recovery rate (%) of auxins (IAA, NAA and IBA) by HPLC-MS/MS.

Samples	IAA (ppb)	NAA (ppb)	IBA (ppb)
Spike 1	84.23	406.1	200.5
Spike 2	88.1	466.5	146.3
Spike 3	250.4	433.4	285
Average	141	435.3	210.6
<b>Recovery rate (%)</b>	<b>28.27</b>	<b>87.1</b>	<b>42.1</b>

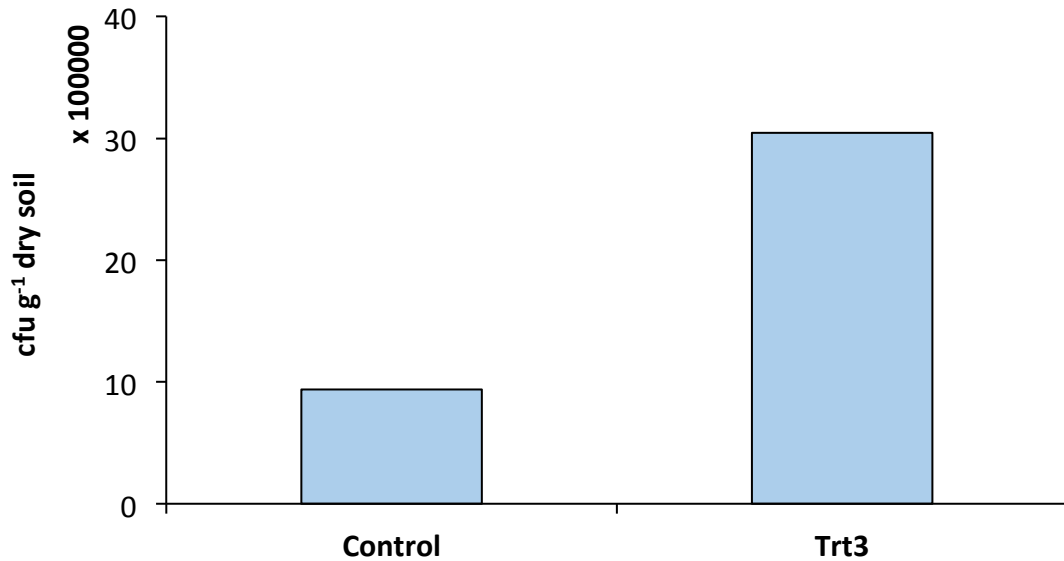


Figure 4.1. Colony forming units (cfu) g<sup>-1</sup> dry soil in late October, 2014 for control and Trt3 (120L ha<sup>-1</sup> yr<sup>-1</sup> Bio-Ag probiotic application) treatments in the greenhouse study. Probiotic was applied in August and early October 2014.

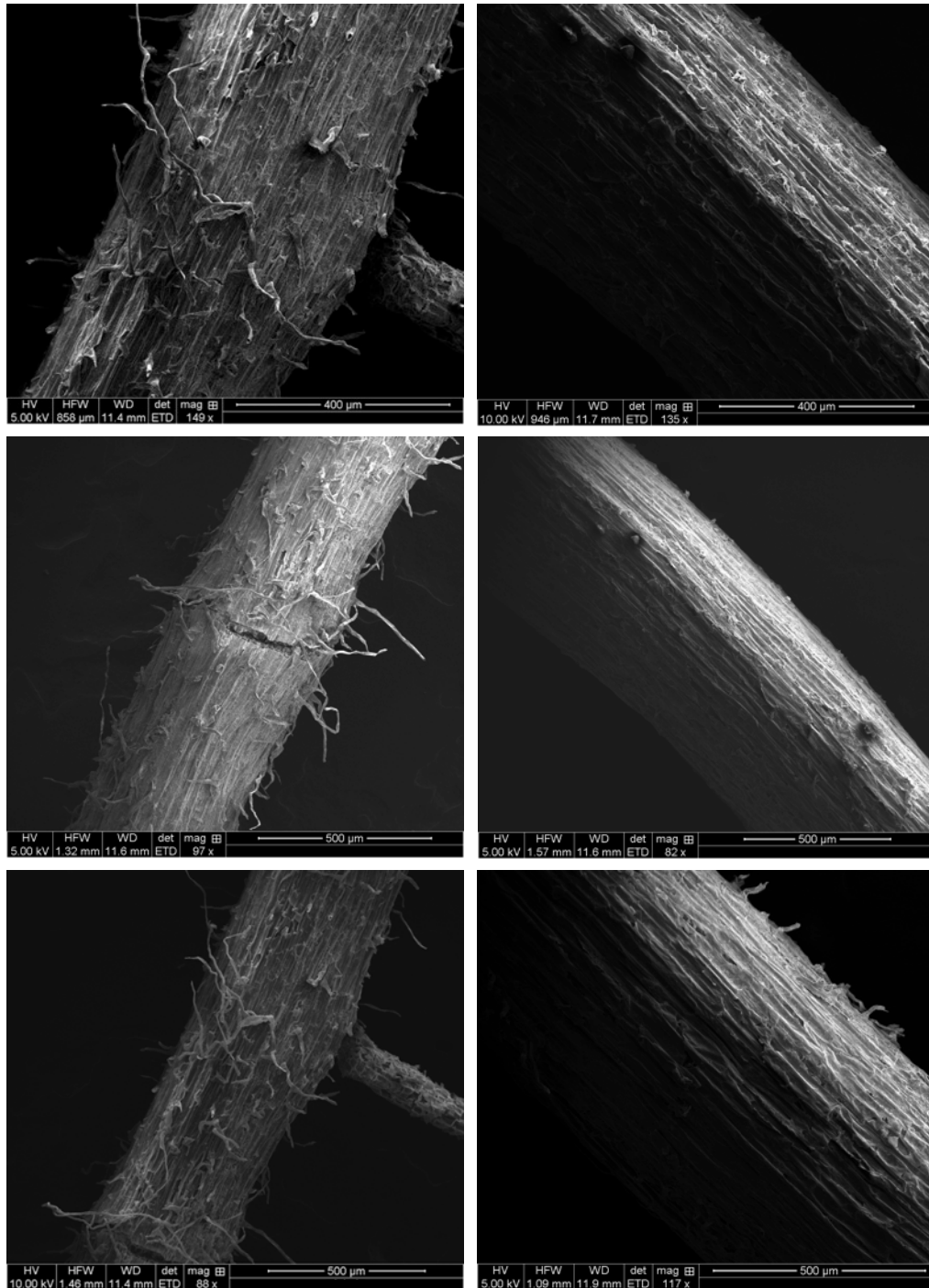


Figure 4.2. Scanning electron microscope (SEM) images of Hairy vetch roots treated with probiotics (L) and control (R) after seven days of first split application of probiotic ( $120\text{L ha}^{-1}\text{ yr}^{-1}$ ) at the University of Missouri-Columbia greenhouse.

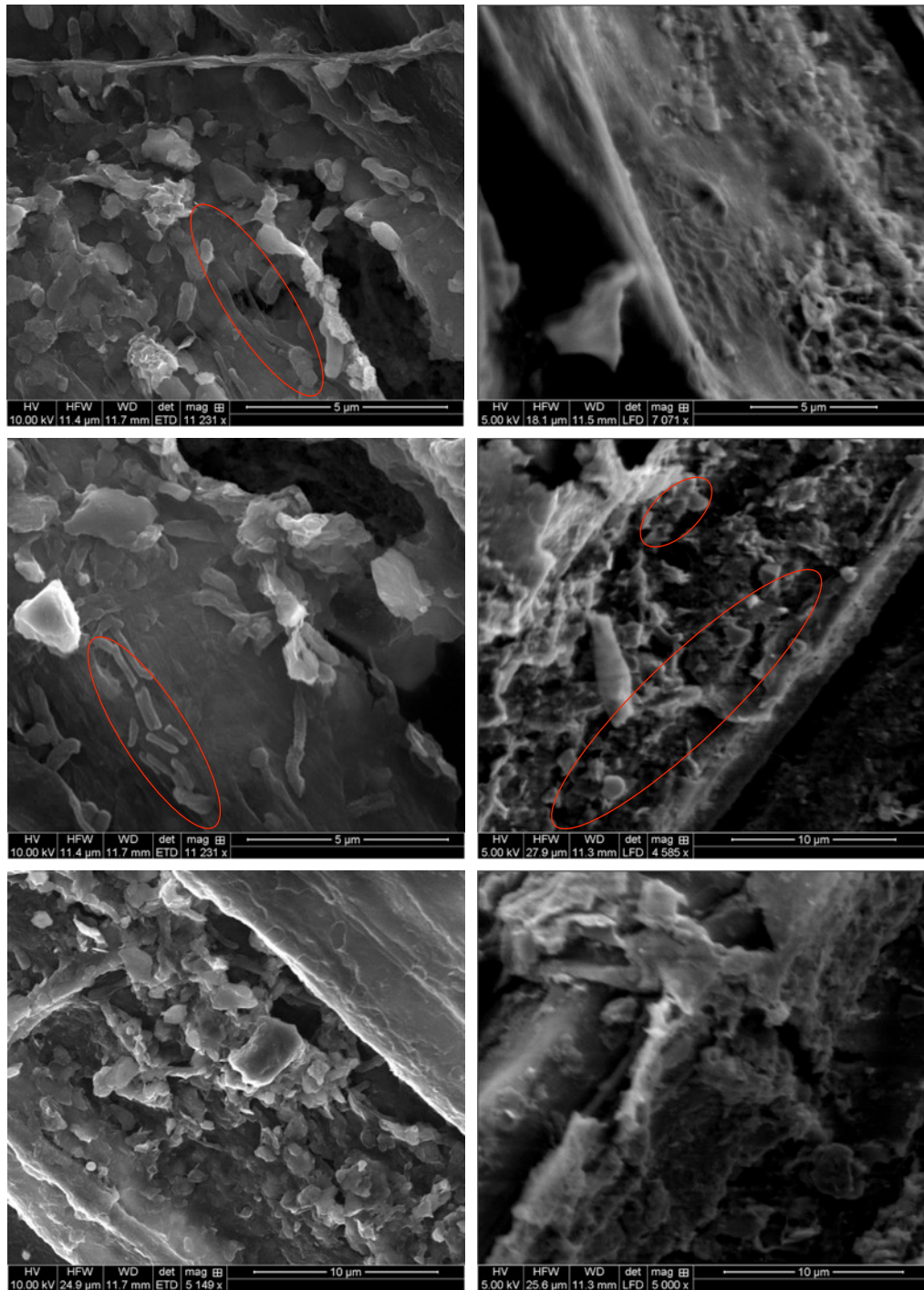


Figure 4.3. Scanning electron microscope (SEM) images showing the rhizobacteria colonization on the hairy vetch roots treated with probiotics (L) and control (R) after seven days of first split application of probiotic ( $120\text{L ha}^{-1} \text{yr}^{-1}$ ) at the University of Missouri-Columbia greenhouse. Image acquisition conditions were different among images.

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## CHAPTER 5

### CONCLUSIONS

Results from the field study suggest that the high concentrations of probiotics ( $120\text{L ha}^{-1}\text{ yr}^{-1}$ ), relative to the recommended dosage, positively correlated with higher soil fungal communities including both AMF and saprophytic fungi. Results also indicated a strong positive correlation of DHA and FDA with all microbial groups respectively. Strong positive correlations were also found between  $\beta$ -glucosaminidase and all microbial groups.

No significant results were observed in total bacterial community although the probiotic solution contains mostly bacteria (gram-positive and gram-negative bacteria). However, a positive correlation between the dosage of probiotic applied and soil bacterial communities was observed.

PCA showed that most of the variance in fatty acid biomarkers in high dose treatment at time one but rhizobia and protozoa fatty acid biomarkers increased at time two only. Our study implies that probiotics promoted soil microbial biomass and diversity at  $120\text{L ha}^{-1}\text{ yr}^{-1}$ .

In the greenhouse study, bacteria biomass increased greatly with time for the probiotic treatment ( $120\text{L ha}^{-1}\text{ yr}^{-1}$ ), which suggests the effect of probiotics varies when temperature and soil moisture is controlled. With the highest concentration of probiotics ( $150\text{L ha}^{-1}\text{ yr}^{-1}$ ), most of the microbial groups decreased including total microbial biomass. Increasing trends for soil fungal communities, rhizobia and protozoa with

increasing probiotic concentrations suggest that higher concentrations of probiotics could be used in future studies to quantify their effects on other soil microbial communities.

The results of the greenhouse experiment also suggest that probiotic promote the microbial density on roots of hairy vetch. Some of the bacillus-like microbial biofilms were observable. Additionally, long-term effects of probiotics on roots can be observed microscopically on samples from soils and plants inoculated for more than 7 days.

Fluorescent pseudomonads were enhanced with probiotic application in the rhizosphere, which are one of the main drivers of auxin and production of many other phytohormones in soil. Results imply indirect and positive effects of probiotics on production of auxin and other phytohormones.

There was no effect of probiotics on auxin (IAA, IBA and NAA) production in the soils up to a 25ppb detection limit. Results may imply that there is no effect of probiotics on auxin content in the soils when no substrate is applied. Moreover, analysis can be done in the future with the HPLC-MS/MS method in presence of artificial precursors of auxin such as TRP.

Our results suggested that probiotics can be used on cover crops to increase soil fungi biomass and DHA activity in the soils which are soil quality indicators. Probiotic increased root hair growth of hairy vetch cover crop and could be used for other crops. Furthermore, similar study can be conducted with high concentration of probiotics and non-treated control for the subsequent crops with addition of other parameters (soil physical and chemical properties, soil erosion, crop yield) to assess soil microbial activity and its relative effect on other soil parameters and crop yield.

## APPENDIX

A. Physicochemical properties of soils at Soil Health Demonstration Farm, Chariton County, Missouri, U.S.A collected on 02-14-2013 from 0 to 15cm depth.

Variables	
Organic matter (%)	2.5
CEC (meq/100g)	16.7
pH (water pH)	6.1
Neutralizable acidity (meq/100g)	2.35
Phosphorus; Bray 1 (ppm)	10
Phosphorus; Bray 2 (ppm)	12.5
Potassium (ppm)	118
Zinc (ppm)	0.55
Sulfur (ppm)	10.5
Calcium (ppm)	2266.5
Magnesium (ppm)	325

**B.** Colony forming units count of fluorescent pseudomonads on spread plate at different dilution factors. Time one and two represents after first and second split application of probiotic respectively.

Treatments	Dilution factor		
	0.001	0.0001	0.00001
Time 1	Colony forming units (cfu) g <sup>-1</sup> dry soil		
Control	2.84x10 <sup>5</sup>	9.63x10 <sup>5</sup>	3.35x10 <sup>6</sup>
Trt3 (120L ha <sup>-1</sup> yr <sup>-1</sup> )	4.05x10 <sup>5</sup>	1.27x10 <sup>6</sup>	4.44x10 <sup>6</sup>
Time 2			
Control	2.34x10 <sup>5</sup>	9.41x10 <sup>5</sup>	8.26x10 <sup>6</sup>
Trt3 (120L ha <sup>-1</sup> yr <sup>-1</sup> )	4.39x10 <sup>5</sup>	3.04x10 <sup>6</sup>	1.30x10 <sup>7</sup>