

**IMPACT OF MINERAL N AND P AND MANURE ON
ARBUSCULAR MYCORRHIZAL FUNGI, OTHER SOIL
MICROORGANISMS AND ON SOIL FUNCTIONALITY IN
DIFFERENT AGROECOSYSTEMS**

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ABSTRACT

Microorganisms and their interactions in soil play a critical role in nutrient transformations and cycling, and in sustaining soil productivity. Arbuscular mycorrhizal fungi (AMF) are a keystone group of fungi influencing nutrient cycling. In turn, the activity and composition of microorganisms in soil are influenced by management practices such as the choice of crop species and fertilization. Long-term effects of cropping, manuring and mineral fertilization on the soil-plant system were defined in three selected agro-ecosystems of Canada. A greenhouse experiment was also conducted to define the involvement of AMF in organic residue decomposition and nitrogen (N) mineralization.

In the greenhouse experiment, pasteurized soil was inoculated or not with a strain of *Glomus claroideum*, *G. clarum* or *G. intraradices*. ^{15}N -labelled organic residue in a nylon mesh was buried in the soil. The fate of residue-N was determined after 24 weeks. Arbuscular mycorrhizal fungal species enhanced mineralization of organic residue to different degrees. The highest recovery of mineralized N by plants (25%) occurred when inoculated with *G. clarum*. The AMF enhanced N-mineralization differentially leading to differential plant growth stimulation, differences in the C-to-N ratio of the decomposing organic residue, and in soil microbial community structure.

In a field trial conducted on a Brown Chernozemic soil at the Semiarid Prairies Agricultural Research Centre in Swift Current, SK, eight years of phosphorus (P) (0, 20 and 40 kg $\text{P}_2\text{O}_5 \text{ ha}^{-1}$) application to alfalfa monoculture and alfalfa-Russian wildrye (RWR) dual culture modified the soil microbial community structure. Low levels of phosphorus (0 and 20 kg $\text{P}_2\text{O}_5 \text{ ha}^{-1}$) fertilization in alfalfa-RWR dual culture increased

the abundance of fungivorous nematodes and grazing of AMF hyphae thus increasing the carbon drain from plants and ultimately reducing plant biomass.

In a sub-humid region of Saskatchewan on a Black Chernozem soil, mineral N (0, 20 or 40 kg N ha⁻¹) was applied for 10 years to pea grown continuously or in rotation with wheat. Lower yields in continuous-pea were associated with reduced abundance of beneficial Gram positive bacteria and AMF, and an increase in uptake of plant available Fe to toxic levels. These differences in soil properties were related to root rot which increased with years in continuous-pea. The soil environment in the continuous-pea rotation further led to lower organic carbon inputs, and to reduced soil microbial biomass and soil enzyme activity indicating a negative impact on nutrient cycling.

In the south coastal region of Agassiz, British Columbia, dairy manure slurry (DMS) and ammonium nitrate (AN) had been applied on a Regosol at the same annual rate of mineral N (50 or 100 kg mineral N ha⁻¹) for nine years to perennial tall fescue, followed by one year of stand renovation through reseedling without fertilization. The multi-year application of DMS improved soil organic C, soil organic N, light fraction of organic matter, microbial biomass and enzyme activity as compared to mineral fertilization but the DMS-related increase in soil yield potential was lost in the process of stand rejuvenation. Dairy manure slurry application based on the crop N requirement also increased soil phosphate indicating increased environmental hazard. In conclusion, long-term use of DMS in multi-cut tall fescue can increase soil quality parameters but can also increase the risk of eutrophication of water bodies.

Overall, data showed that higher levels of soil nutrients can select for certain bacteria while AMF and other bacteria are more abundant under low soil fertility. On the

other hand, different soil microbial groups were associated with different soil enzyme activities. From this study, I succeeded in proving my hypothesis that practice of fertilization and choice of crop influence soil microbial community structure which further affect soil functioning.

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DEDICATION

I dedicate my thesis to my

Parents

Kusam and Vijay Kumar Nayyar

whose efforts and ever willing support have made this dream come true.

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LIST OF ABBREVIATIONS

AEM	Anion exchange membrane
AMF	Arbuscular mycorrhizal fungi
AN	Ammonium nitrate
C	Carbon
Ca	Calcium
DMS	Dairy matter slurry
DW	Dry weight
ERM	Extraradical mycelium
FAME	Fatty acid methyl ester
Fe	Iron
HLD	Hyphal length density
IRM	Intraradical mycelium
LFOM	Light fraction organic matter
MBC	Microbial biomass carbon
Mg	Magnesium
MHB	Mycorrhiza-Helper-Bacteria
Mn	Manganese
N	Nitrogen
NLFA	Neutral lipid fatty acid
OR	Organic residue
P	Phosphorus

PLFA	Phospholipid fatty acid
PSB	Phosphate-solubilizing bacteria
RWR	Russian wildrye
TC	Total carbon
TON	Total organic nitrogen
TPF	Triphenyl formazone

1. INTRODUCTION

Soil microorganisms are important components of agricultural ecosystems. They are involved in sustaining soil productivity through organic matter decomposition and nutrient transformations and cycling. Microorganisms regulate the nutrient flow in the soil by assimilating nutrients and producing biomass and converting organically bound forms of nutrients, especially C, N and P, to mineral forms (Wani and Lee, 1995). The role of the soil microbial resources existing in soil is still being explored. Because of the enormous importance of plant-microorganism interactions in the soil for ecosystem functioning and nutrient cycling, it is critical to identify the factors influencing the microbial communities in this habitat.

Fertilizers are added to soil primarily to increase nutrient availability and crop productivity. Inorganic fertilizers, especially N and P, when applied continuously can directly or indirectly affect soil biological properties which, in the long run, can affect the quality and productivity of soils (Acton and Gregorich, 1995). However, conflicting results about the effect of long-term application of inorganic fertilizers on soil microbial biomass and diversity exists in the literature. Some studies showed that inorganic fertilizers increase soil microbial biomass (Kanazawa et al., 1988; Goyal et al., 1992) but Sarathchandra et al. (2001) reported that inorganic fertilizer had no effect on microbial population and that N application reduced microbial diversity in soils. On the other hand, application of organic manures increased soil C which increased microbial biomass and modified soil microbial community structure (Sun et al., 2004). In comparative field studies where manure and fertilizers were applied, Mäder et al. (2002) reported greater increase in microbial activity, microbial biomass and root colonization by arbuscular

mycorrhizal fungi (AMF) in organic farming systems than in conventional ones.

Hartmann et al. (2006) also showed that bacterial communities were mainly influenced by the presence or absence of organic fertilizer, irrespective of conventional or organic farming management. Knowledge about the shifts occurring in microbial community structure and diversity following different agricultural management practices could improve our knowledge of biological processes and nutrient cycling in cultivated soils.

1.1 Objectives

Based on the above mentioned information, four objectives were defined:

- To define the impact of P fertilization, a widespread practice in crop production, on soil microorganisms and soil functioning through the examination of two crops in an 8-year old field experiment which was available in Swift Current Saskatchewan.
- To define the long-term effect of N fertilization on soil microorganisms and soil functioning through the examination of two crop rotations in 9-year old field experiment established in Indian head, Saskatchewan.
- To compare the effect of organic and mineral fertilizers on the soil microbial community and soil functioning through the examination of a 10-year old field experiment involving a hay crop located in Agassiz, British Columbia.
- To clearly demonstrate in the greenhouse that soil microorganisms can have a large and direct impact on soil functioning. Although field experiments are most relevant to demonstrate fertilizer effects on soil microorganisms, the complex interactions taking between soil microorganisms and the soil environment and the impossibility to control factors in the field prevent any demonstration of direct

microbial effects on soil functioning. I chose to demonstrate the large and direct effect of AMF on soil functioning.

1.2 Hypotheses

- The greenhouse experiment was designed to test that the effects of AMF on residue decomposition are (a) widespread among AMF species, (b) important for plant N uptake and (c) influential on microbial community structure.
- In the field experiments it was hypothesized that long-term fertilizer regimes and choice of crops and rotations would affect AMF and overall soil microbial community structure thereby potentially influencing soil functioning.

Long-term experiments have an advantage in addressing questions about the effect of cropping practices on the soil system. A primary attribute of such experiments is their longevity which clearly reveals the long-term impact of fertilizers on the soil system. The examination of the effects of fertilization in three experimental sites using the same protocols to measure the same variables allowed the identification of fertilization impacts that are consistent among different agroecosystems.

My studies show that fertilization modifies the soil microbial community structure, and that beneficial microorganisms such as AMF are favoured by low N and P fertility. Microbial interactions also vary with ecosystems. For example, nematodes antagonized AMF in the presence of Russian wildrye and the proportion of beneficial and pathogenic microorganisms changes with crop rotation. The AMF were found to be important in plant N uptake from organic source. The results

indicate that soil microorganisms can be manipulated to benefit soil functioning and plant productivity.

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2. LITERATURE REVIEW

2.1 The Living Soil System, an Overview

Soil is a dynamic, living, natural body consisting of a mineral fraction, obtained from weathered rocks, reactive and stable forms of organic matter that are derived from the remains of plants, animals and microorganisms, water and gases. Mineral and organic particles are intimately bound in soil, forming various types of soil aggregates. Soil organic matter is critical to the maintenance of soil structure, which provides drainage, water-holding capacity and aeration for crop growth and microbial activity. Soil sustains an immense diversity of microorganisms which play a pivotal role in the cycling of nutrients and in the maintenance of soil quality. The quality of soil, therefore, depends not only on its physical and chemical properties but also on the diversity and activity of its biota (Doran and Linn, 1994).

Soil is a storehouse of nutrients and energy for organisms living in soil including plants. Transformations of nutrients in forms that can be used by plants are carried out by a diverse group of soil organisms including nematodes, protozoa, springtails, mites, earthworms, millipedes, fungi, bacteria, archaea and cyanobacteria. Plant life is the primary energy source that drives terrestrial soil ecosystems. Plant residues and roots release various organic materials into the rhizosphere and these carbon-containing substances act as a fuel for the growth of the microbial community. Plants thus play an important role in influencing the number, diversity and activity of microorganisms and

the interactions among these, plant root and the soil. The microbial community in the vicinity of roots can ultimately influence health, vigor and productivity of the plant.

2.2 Major Soil Processes Mediated by Soil Microorganisms

The presence of organisms in soil makes the soil a living system. Soil organisms contribute a wide range of essential services to the sustainable functioning of all ecosystems regulating the chemical equilibrium of the Earth. In particular, they act as the driving agents of nutrient cycling, regulating the dynamics of soil organic matter and soil carbon sequestration, modifying soil physical structure and enhancing plant health. Some of the important soil processes regulated by microorganisms are described below.

2.2.1 Organic matter decomposition

Decomposition of organic matter is an important microbial process in nutrient cycling where complex organic compounds are physically and/or enzymatically broken down into forms easily assimilated by plants. In agricultural ecosystems, crop residue is the primary C substrate for the microbial community. When the plant residues are returned to the soil, macro and mesofauna break complex organic molecules into simpler organic and soluble molecules (Juma, 1998). The simpler organic materials produced during faunal fragmentation are further subjected to decomposition by microflora. The extracellular and intracellular enzymes produced by microorganisms hydrolyse various bonds that hold nutrients in organic combinations, thus releasing mineral nutrients into soil, a process known as mineralization (Whalen and Hamel, 2004).

During the decomposition of plant residues, a portion of its carbon is mineralized into carbon dioxide and released into the atmosphere, which is a major factor in the carbon cycle, and another fraction is incorporated into microbial biomass. As the plant

residue and microbial biomass undergo further decomposition and biochemical alteration, other compounds are synthesized by microorganisms. Some of these compounds react and form complex humic materials that represent only a small percentage of the soil volume, but exerts a great influence on the physical, chemical and biological properties of the soil.

Decomposition rates vary with the amount, physical size and chemical composition of the organic material, the types of soil organisms present, and the prevailing environmental conditions (Lee and Pankhurst, 1992; de Ruiter et al., 1994). Microbial biomass and mineralization are also influenced by protozoa and nematodes feeding on microbes (Griffiths, 1994; Ferris et al., 1998; Bonkowski et al., 2001). They function as secondary consumers, feeding largely on bacteria and fungi, thereby increasing the rate of turnover of microbial biomass and the associated nutrients. About 70% of the consumed microbial biomass is excreted into soil (Whalen and Hamel, 2004). Nematodes have a higher C to N ratio than the bacteria and fungi they feed upon and are a very important factor for N mineralization. Increases in N mineralization rates with increases in bacterivorous nematodes have been reported in grassland (Hassink et al., 1993), riparian (Ettema et al., 1998) and coniferous forest (Forge and Simard, 2001) ecosystems. Earthworms also influence nutrient cycling by consuming organic matter and grazing on soil microbes. Organic matter (including roots, algae, bacteria and fungi) in the range of 2 to 15 Mg ha⁻¹ yr⁻¹ has been reported to be consumed by earthworms (Whalen and Parmelee, 1999). The portion of organic matter not utilized by earthworms is released through their casts and guts. Microorganisms decompose the cast of earthworms which acts as a source of nutrients for them (Tiunov and Scheu, 2000).

2.2.2 Nitrogen cycle

Nitrogen is an essential nutrient for all forms of life on Earth as it is a key component of proteins, nucleic acids and other cell constituents. The cycling of N among its many forms is a complex process by which its atoms cycle between the atmosphere and the biosphere. In general, the N cycle comprises the processes of fixation, assimilation, ammonification, nitrification and denitrification (Fig. 2.1).

Although a small amount of N is fixed by lightning in the atmosphere (Lelieveld and Dentener, 2000) and introduced into ecosystems largely over tropical continents, most N₂ fixation is mediated by some prokaryotes, of the bacteria, cyanobacteria and the actinomycete *Frankia*, and some archaea. In the process of N₂ fixation, microorganisms possessing nitrogenase break the triple bond linking two atmospheric N atoms, reducing them to ammonia, a plant available form of N. Nitrogen-fixing microorganisms, also called diazotrophs, can exist as free living (Kennedy and Islam, 2001), associative (Steenhoudt and Vanderleyden, 2000) or symbiotic organisms (Tajima et al., 2000; Wall, 2000). The best-studied example of symbiotic N fixation is the association between legumes and rhizobial bacteria in the genus *Rhizobium*, *Bradyrhizobium* and *Mesorhizobium*. Rates of symbiotic N fixation in legumes vary with host, microsymbiont and environment. Nitrogen fixation of 50 to 200 kg ha⁻¹ a⁻¹ has been reported in forage legumes (Wedin and Russelle, 2007).

Plant use inorganic N, either fixed by symbiotic and nonsymbiotic bacteria or anthropogenically added through fertilizers, to produce vital cellular products such as proteins. The process is termed assimilation (Fageria, 2009a). Nitrate assimilation, however, requires that nitrate be first reduced to ammonium before its incorporation into

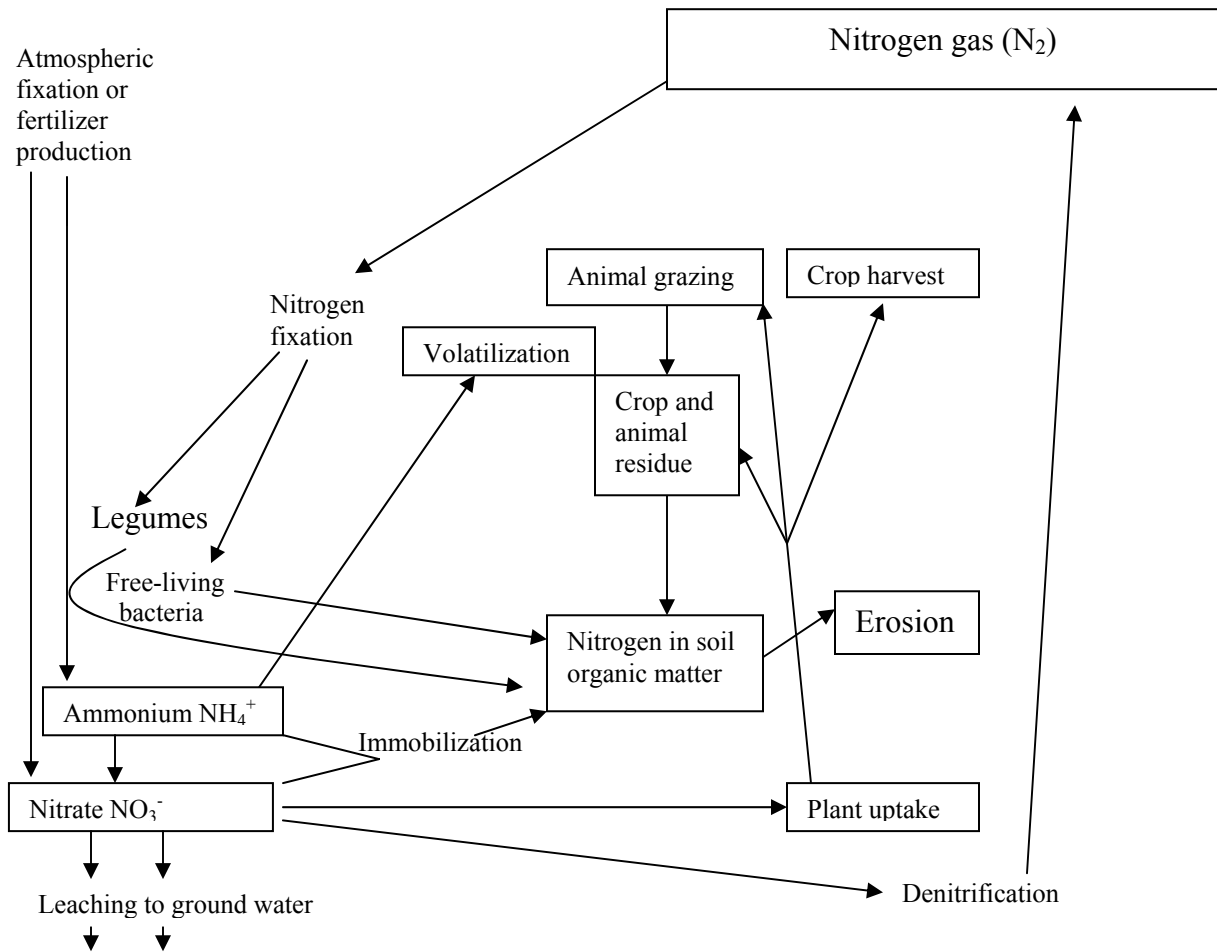


Figure 2.1 Nitrogen cycle

cell constituents. The role of AMF in N assimilation by plants is discussed in Section 2.5. There are also reports of AMF assimilation amino acids (Hawkins et al. 2000). The plants are then eaten by animals to meet their protein requirements. The plant material and the animal wastes are returned to soil and serve as an important substrate for the heterotrophic soil microorganisms. The conversion of complex organic-nitrogen compounds to ammonium is termed ammonification (Fageria, 2009a) and is mediated by a wide variety of enzymes produced by heterotrophic soil microorganisms (Prasad and Power, 1997). The process involves both extracellular and intracellular enzymes (Myrold, 2005). Extracellular enzymes first degrade organic-nitrogenous polymers to monomers. The monomers are further metabolized by intracellular enzymes within the microbial cell resulting in the production of ammonium which is released into soil solution. This ammonium is again available for either assimilation by plants and microorganisms or conversion to nitrate by the nitrifiers.

Nitrification is a two step microbial process in which $\text{NH}_3/\text{NH}_4^+$ is first converted to nitrite by the ammonia oxidizing bacteria of the “Nitroso” genera through activities of ammonia monooxygenase and hydroxylamine oxidoreductase (Prosser, 2006), and then nitrite is oxidized to nitrate by the nitrite-oxidizing bacteria of the “Nitro” genera (Ye et al., 1994) using nitrite oxidoreductase (Prosser, 2006). The ammonia and nitrite oxidizers are chemoautotrophic bacteria that obtain their energy from these inorganic oxidations while using carbon dioxide as their source of carbon. Soil nitrification can also be carried out by heterotrophic bacteria and fungi but their activity is lesser than that of autotrophs (Prosser, 2006).

The nitrates so produced have many fates. Most plants and microorganisms can take up nitrates but these are first reduced to ammonium before incorporation into amino acids. The process of nitrification has, however, some important environmental consequences. Unlike ammonium, the negatively charged nitrate is not held on soil and organic matter particles and can easily be washed off into either ground or surface water. In this way, not only the valuable N is lost from the soil, reducing soil fertility, but also causes the problems of nitrate toxicity and eutrofication. Intensive use of fertilizers to raise crops enhances these problems. Nitrates can also undergo reduction by denitrifying bacteria which convert nitrate into gaseous N species, generally dinitrogen and nitrous oxide and thus nitrogen enters back to the atmosphere. Among the many factors that influence the denitrification process, soil water content, carbon availability and soil nitrate concentration are commonly identified as the most common factors controlling denitrification (Weier et al., 1993; Gillam et al., 2008). Human activities are increasingly altering the N global cycle through the production and use of N fertilizers, and the combustion of fossil fuels to meet the higher demand of energy, food and industry. The effect of anthropogenic N additions on the N cycle at the global and regional scales has been addressed by Galloway et al. (2004, 2008).

2.2.3 Phosphorus cycle

Phosphorus is an essential nutrient for plants and animals. Phosphorus does not enter the atmosphere, remaining mostly on land and in rock and soil minerals (Stevenson and Cole, 1999). In contrast to N whose supply from the atmosphere is virtually unlimited, the ultimate source of all soil P is primary apatites. These apatites undergo slow weathering by simultaneous pedologic and biologic pathways (Smeck, 1985). Under the influence of

weathering processes, soluble P (orthophosphate) is released into soil solution which may attain equilibrium with the soil labile P pool. The soluble P may be taken up by plants or other organisms, be leached out of the soil, or be incorporated into secondary P minerals (Fig. 2.2) (Fageria, 2009b). With time, the P in the secondary minerals is slowly transformed into occluded P, a pedologic P sink. Soil P may also find its way to surface waters through run off and erosion and ultimately to oceans where it is deposited as sediments.

In a biological P cycle, soluble P taken by plants and organisms is returned back to soil as plant residues, animal remains and wastes where it is again mineralized to orthophosphate for another biological cycle, or immobilized in secondary P forms, or incorporated into stable organic soil P. While weathering of minerals, desorption from labile P pool and mineralization release P into soil solution, the process of adsorption and precipitation of soluble P and immobilization by microorganisms decrease soil solution P. Increasing P levels in soil elevate the potential P runoff to aquatic ecosystems (Fluck et al., 1992). Production in most lakes depends on P input (Schindler, 1977). Over enrichment with nutrients can cause excessive production of algae in lakes, a problematic condition known as eutrophication, in which water quality is impaired. Therefore, P recommendations and applications should balance the plant demand and P losses.

2.3 Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi belong to the phylum Glomeromycota (Schüßler et al., 2001) and form symbiosis with about 250,000 species of plants worldwide (Smith and Read, 1997). They propagate in soil as spores, hyphae, or colonized root fragments. During the plant-AMF symbiosis, the fungus penetrates the root cortical cell walls and

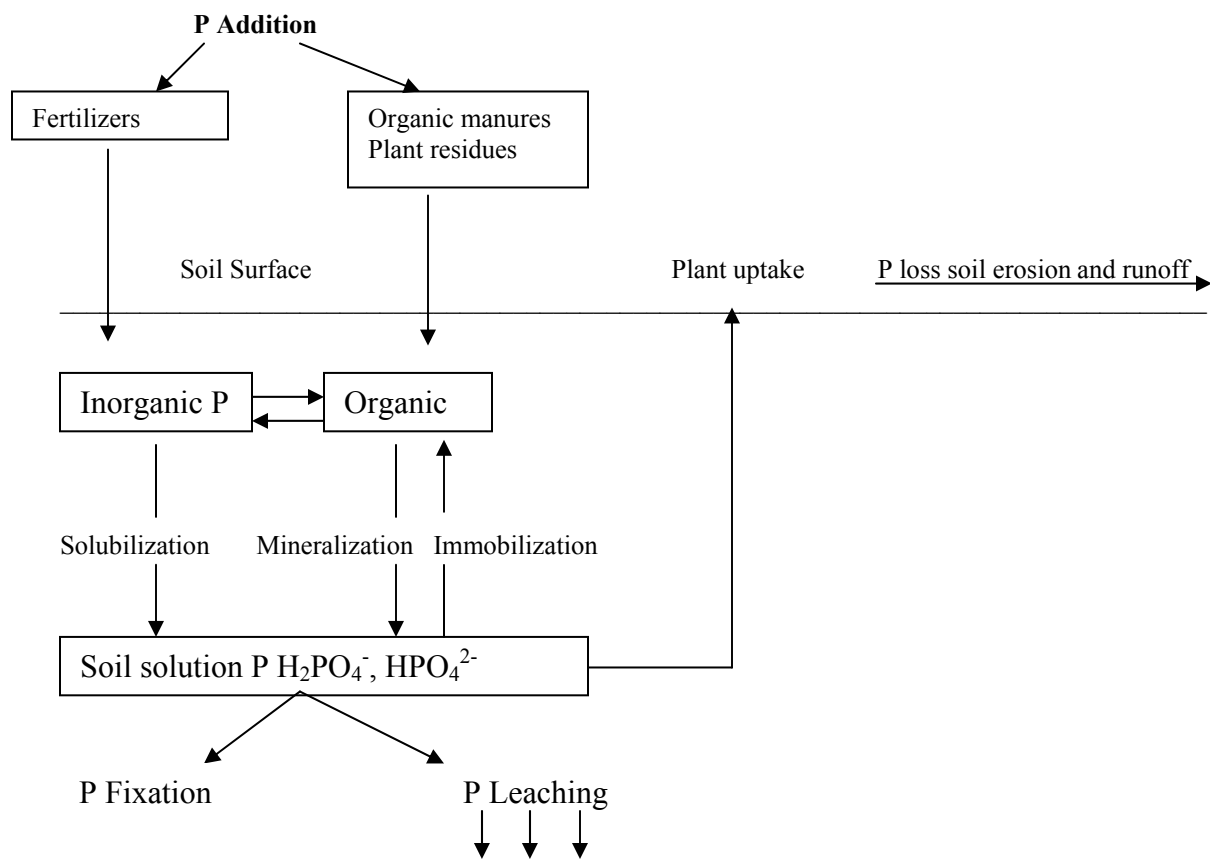


Figure 2.2 Phosphorus cycle

forms highly branched intracellular fungal structures called arbuscules. Arbuscules are specialized hyphae formed as intercalary structures between coil hyphae. They are thought to be the main site of nutrient exchange between fungus and plant (Hodge, 2000). The hyphae of AMF provide roots with increased surface to explore a greater soil volume for nutrients and water.

Arbuscular mycorrhizal fungi influence plant growth in a number of ways (Klironomos, 2003). The extraradical hyphae of AMF extend several centimetres into the soil and help the plants in uptake of mineral nutrients especially the immobile nutrients such as P, Zn and Cu (Marschner and Dell, 1994). Their role in N uptake has also been demonstrated (Subramanian and Charest, 1998, 1999; Toussaint et al., 2004; Govindarajulu et al., 2005; Atul-Nayyar et al., 2009). The hyphae of AMF also play an important role in stabilization of soil aggregates (Wilson et al., 2009). The soil fungal mycelium entangles soil particles within the hyphae network and cements particles together through exudation of extracellular polysaccharide compounds such as glomalin (Rilling, 2004; Treseder and Turner, 2007). Glomalin stores carbon in both its protein and carbohydrate (glucose or sugar) subunits. It permeates organic matter, binding it to silt, sand, and clay particles which is described as a major factor in the formation of soil aggregates (Bossuyt et al., 2001; Miller and Jastrow, 2002). Besides, AMF also enhance plant resistance to pathogens (Lingua et al., 2002) and tolerance to environmental stresses (Newsham et al., 1995).

2.4 AMF and Phosphorus Uptake

Phosphorus typically constitutes between 30% and 65% and sometimes 80% of the total P in soil as organic P (Fageria, 2009b). Phosphorus moves by diffusion in soil

and is taken up by plants through root interception. Soil P availability typically is low and plants form symbiosis with AMF to maintain optimum growth. The extraradical hyphae of AMF act as root extensions and draw P from soil to supply it to plants (Zapata et al., 2009). There is a large body of literature reporting increase in P concentration in mycorrhizal plants (Smith and Read, 1997; Pasqualini et al., 2007; Yao et al., 2008). However, growth and/or yield responses to AMF could range from negative to positive depending on the development stage and soil P availability (Li et al., 2005). In a short-term ^{32}P compartmentalized pot experiment, Li et al. (2008) showed that *Glomus intraradices* could deliver 50% of the P that plants received from soil, even though there were no positive growth responses.

The P benefits from AMF to plants are influenced by the source and amount of P additions in soil. Soluble P fertilizers increase its concentration in the soil solution and plant tissue, and thereby decrease the mycorrhizal association (Vierheiling, 2004). Less soluble P fertilizers like rock phosphate have less effect as P concentration in the plant and may not reach a level that tend to inhibit mycorrhizal association (Toro et al., 1997). Organic manures, however, generally enhance AMF formation and fungal propagules (Harinikumar and Bagyaraj, 1989; Muthukumar and Udaiyan, 2002). Stimulation of AMF by organic matter may be due to release of available nutrients or to the presence of certain molecules (Gryndler et al., 2009). The role of organic amendments may be of particular importance in agroecosystems where these are used regularly as a surrogate to synthetic fertilizers or in organic agriculture. In a long-term experiment, Joner (2000) reported that mycorrhizal formation was the highest in soil that had received no P for 74 years, followed by a soil having received 30 or 60 Mg ha⁻¹ farmyard manure then soil

with 25 or 44 kg P ha⁻¹ in mineral fertilizers. The author also observed that moderate quantities of farm yard manure had less of an adverse effect on AMF than equivalent amounts of nutrients in NPK fertilizers which may be due to the gradual release of P from the organic source balancing plant demand.

Soil water conditions also affect AMF development and P uptake. Jayachandran and Shetty (2003) studied the impact of AMF on growth and P uptake of wet prairie saw grass (*Cladium jamaicense* Crantz). They observed that root and shoot growth and P uptake were increased in mycorrhizal plants as compared to control plants, and decreasing water content were conducive to AMF development. Neumann and George (2004) concluded that total plant P content is similar in mycorrhizal and non-mycorrhizal plants under well-watered conditions but P uptake is twice as high as in mycorrhizal plants as compared to non-mycorrhizal plants when the soil is dry.

2.4.1 Mechanisms of P transfer from fungus to plant

Plants take up inorganic P (P_i) mainly as orthophosphate ions dissolved in the soil solution or adsorbed on the surface of soil particles (Holford, 1997). Inorganic P can be absorbed by AMF plant both directly at the soil-root interface through root epidermis and root hairs and via the mycorrhizal pathway through external AMF hyphae in soil. Uptake of P directly from soil decreases as the concentration of P in the soil solution adjacent to the roots decreases, thereby creating a depletion zone around the roots. The ability of extraradical hyphae to grow beyond this depletion zone and to deliver P to the root is thought to be the main basis for their positive effects on P uptake and plant growth (Smith and Read, 1997). Owusu-Bennoah and Wild (1980) reported that the depletion zone around mycorrhizal onion roots is twice as large as around non-mycorrhizal roots.

The ERM of AMF expresses Pi transporters genes such as GvPT, GiPT and GmosPT and absorbs P from soil. Once Pi enters the fungal cytoplasm, it accumulates quickly in the vacuole (Solaiman et al., 1999; Ezawa, 2001) and forms polyphosphates (poly-P) (Ezawa et al., 2004). Poly-P accumulated in the vacuolar compartment can be translocated from the ERM to intraradical mycelium (IRM) possibly via cytoplasmic streaming and/or motile tubular vacuole system (Smith and Read, 1997; Olsson et al., 2002). Poly-P is then hydrolysed in the IRM and transferred to the periarbuscular apoplastic compartment from where it is available to the plant.

The increase in uptake of P by mycorrhizal plants has been more pronounced in P limited soils than P sufficient soils (Smith and Read, 1997; Joner, 2000). Hattingh et al. (1973) found that mycorrhizal plants have access to P at greater distances than non-mycorrhizal plants. Smith et al. (2003) reported that the contribution of P transfer via the mycorrhizal pathway is extremely high, the extent varying with fungal species and the plant involved, the symbiosis apparently shutting down the direct Pi uptake system in plants.

The importance of AMF in P mineralization from organic sources, which can be a major source of P for plants in some soils, has not been extensively studied. It was reported that AMF proliferate in organic matter (St. John et al., 1983) and may help in the recycling of mineral nutrients but only a few investigations have considered the utilization of organic P compounds by AMF plants (Joner and Jakobsen, 1994; Cade-Menun and Berch, 1997). Both plants and microbes have the ability to release a group of enzymes called phosphatases or phytases which hydrolyse organic P into inorganic P (Tarafdar and Marschner, 1994; Hayes et al., 1999). From a divided pot experiment,

Tarafdar and Marschner (1994) reported higher acid phosphatase activities in the hyphal compartment of mycorrhizal than non-mycorrhizal plants. A strong correlation between phosphatase activity and AMF hyphal length provided evidence that the enzyme is produced by the fungal symbiont rather than by the background microflora. They also observed that of the total P uptake by plant, the mycorrhizal contribution accounts for 22 to 33% when P was supplied in inorganic form and 48 to 59% in organic form. Under monoxenic conditions, Koide and Kabir (2000) reported that extraradical hyphae of *G. intraradices* were capable of hydrolysing exogenously supplied organic P sources. In another study, Joner et al. (2000) also showed that an AMF were capable of mobilizing and transferring ^{32}P from an organic compound to plants. Whereas AMF have some hydrolytic activity they may also stimulate microbial decomposers (Atul-Nayyar et al., 2009).

2.5 AMF and Nitrogen Uptake

The contribution of the AMF symbiosis to plant P uptake has been well documented, but it is still being debated in the case of plant N uptake. Plants take up N as nitrate (NO_3^-) and ammonium (NH_4^+) from the substrate or from the exchange sites (Toussaint et al., 2004). Also, plants take up a fraction of their N requirement from organic N (Kielland et al., 2006), but NH_4^+ and NO_3^- are the predominant mineral forms of N in agricultural soils and their assimilation is very important for plant growth (Aanderud and Bledsoe, 2009). As inorganic N is highly mobile in soil, it is considered that the N benefit to the plant derived from a mycorrhizal interaction is not important. However, it was shown using an in vitro system that the mycelium of AMF takes up inorganic N from soil and first incorporates it into arginine (Govindarajulu et al., 2005). Although other amino acids

such as glutamine and lysine are present, arginine is the main amino acid in the extraradical mycelium (ERM) where it is transferred, to the intraradical mycelium (IRM) and finally to the plant (Govindarajulu et al., 2005). Toussaint et al. (2004) also reported uptake, transfer and assimilation of $^{15}\text{NH}_4^+$ by AMF in monoxenic cultures. There is evidence suggesting that the uptake (Ames et al., 1983a; Bago et al., 1996) and assimilation (Smith et al., 1985) of N by AMF can play an important role in plant acquisition of less mobile forms of N, for example, organic N (Ames et al., 1983a) ammonium adsorbed on to clay particles (Johansen et al., 1994). Several studies related to increase in N uptake in AMF colonized plants have been reported (Johansen et al., 1993; Tobar et al., 1994; Subramanian and Charest, 1997, 1998). Nitrate uptake enhancement by AMF colonization was supported in a study conducted by Faure et al. (1998) who reported larger $^{15}\text{NO}_3$ uptake in AMF ryegrass as compared to control plants. Cruz et al. (2004) investigated the effect of AMF colonization on N uptake and suggested that AMF colonization improves the N nutrition of plants primarily under low levels. Elwan (1998) observed that N uptake was higher in mycorrhizal than non-mycorrhizal wheat plants. Other studies have reported that hyphae can absorb and transport inorganic N from the soil and to the roots of a host plant (Johansen et al., 1996; Subramanian and Charest, 1998, 1999). Ames et al. (1983b) showed there was significant correlation between celery plant N uptake and percent AMF colonization and total hyphal length per gram of soil. They further stated that AMF-mediated N uptake by plants is greater when N is in inorganic forms. Feng et al. (2002) added ^{15}N labelled urea to study the impact of two species of AMF on N uptake at four P fertilization levels. They observed that inoculation with AMF improved the growth and N content of cotton plants. Hodge et al.

(2001) suggested that *Glomus hoi* decomposes and takes up N directly by decomposition of organic matter (grass leaves).

There are however reports that AMF do not help in N uptake from soil. Hawkins et al. (2000) found that AMF did not increase plant N concentration. They also observed that N deficiencies reduced the percentage root length colonized, hyphal length, total ^{15}N uptake by hyphae and dry weight of mycorrhizal plants.

2.5.1 Mechanisms of N transfer from fungus to plant

The process by which N may be transferred from AMF to host plants has been recently studied. Early research suggests that amino acids may be the preferred form of transport. Subramanian and Charest (1998; 1999) reported higher glutamine synthetase, nitrate reductase and glutamate synthase in maize colonized with *G. intraradices*, suggesting that the AM fungus assimilates both NO_3^- and NH_4^+ . Based on results using monoxenic cultures of carrot roots colonized with AMF, Toussaint et al. (2004) reported that carrot roots took up $^{15}\text{NH}_4^+$ more efficiently than $^{15}\text{NO}_3^-$ and that the extraradical hyphae transferred $^{15}\text{NH}_4^+$ to host roots from the hyphal compartment but did not transfer as much $^{15}\text{NO}_3^-$. They also reported greater NH_4^+ uptake by mycorrhizal roots and suggested NH_4^+ uptake and transfer as the mean of N translocation to plants.

Govindarajulu et al. (2005) explained the N uptake mechanism by AMF and its translocation to host plant. They showed that inorganic N is taken up by AMF from substrate and mainly incorporated into arginine, the main amino acid in the ERM. It is transferred, to the IRM and finally to the plant. Nitrogen assimilation in ERM has been attributed to glutamate synthase and glutamine synthetase in ERM, whereas gene

associated with arginine breakdown is expressed in IRM, suggesting release of ammonium to host plant.

2.6 Interaction of AMF with Other Microorganisms

Soil organisms interact with each other and play important roles in soil functioning. Among microorganisms, bacteria and fungi are found to be greatly diverse in soil and most of the studies related to microbial interactions have focused on these microorganisms (Bowen and Rovira, 1999). The microbial population may interfere with or benefit the establishment of mycorrhizal symbioses. Garbaye (1994) used the term 'Mycorrhiza-Helper-Bacteria' (MHB) for those bacteria known to stimulate mycelial growth in mycorrhizal fungi and/or enhance mycorrhizae formation. Soil microorganisms increase root exudation, stimulating AMF hyphal growth in the rhizosphere and increasing root penetration by the fungus. Some biologically active substances such as amino acids, plant hormones, vitamins and other organic compounds released by the soil microorganisms also enhance the growth rate of AMF (Azcón-Aguilar and Barea, 1995; Barea, 1997; Requena et al., 1997; Barea, 2000). Negative impacts of soil microorganisms on AMF have also been reported (Linderman, 1992).

Arbuscular mycorrhizal fungi were shown to directly and indirectly interact with other soil organisms (Atul-Nayyar et al., 2009). Direct interactions include supply of energy-rich C compounds derived from host plants (Johnson et al., 2001), changes of mycorrhizosphere i.e pH induced by the fungus (Filion et al., 1999), competition for nutrients (Hodge et al., 2001) and fungal exudation of inhibitory or stimulatory compounds (Andrade et al., 1997). Indirect interactions include modification of root exudates (Secilia and Bagyaraj, 1987) and soil structure (Bossuyt et al., 2001).

The interaction between rhizobia and AMF has received considerable attention because of the relatively high P demand of N₂ fixation. Since AMF association with plants evolved much earlier than rhizobia-legume symbioses (Provorov et al., 2002), it is believed that legume nodulation may have evolved from already existing AMF association (Gianinazzi-Pearson, 1997). The increase in P uptake facilitated by AMF enhances the functioning of nitrogenase, the enzyme responsible for N₂ fixation in diazotrophic bacteria. The two symbioses typically act synergistically, resulting in greater N and P content than when each symbiosis is inoculated onto the legume alone (Chalk et al., 2006). Since AMF colonization can help plants in coping with drought stress (Augé, 2001; Porcel et al., 2007), the role of this symbiosis in legumes is important. The AMF inoculation has been shown to improve nodulation and N₂ fixation under low water potential (Azcón et al., 1995; Ruiz-Lozano et al., 2001).

Plant-growth-promoting rhizobacteria (PGPR) are a group of bacteria that influence plant growth and crop yield by the production of compounds, such as siderophores (Saikia et al., 2006). Zaidi et al. (2004) reported that dual inoculation of N₂ fixer *A. chroococcum* and AMF *G. fasciculatum*, stimulated plant growth and increased N and P uptake by greengram (*Vigna radiata* L. Wilczek). Khan and Zaidi (2007) demonstrated the benefits of triple inoculation of *A. chroococcum*, *Bacillus* sp. and *G. fasciculatum* on wheat yield, N and P concentrations and quality of wheat grains.

The synergistic microbial interaction between AMF and phosphate-solubilizing bacteria (PSB) in improving P supply to plant has been reported (Barea et al., 2002). Multimicrobial interactions, including those between locally isolated AMF, PSB and *Azospirillum*, a N₂ fixing rhizosphere inhabitant, have also been shown. This indicates

that microorganisms act synergistically when inoculated simultaneously (Muthukumar et al., 2001). Various interactions among different microorganisms including AMF-rhizobia and plant growth promoting rhizobacteria have also been reported (Requena et al., 1997).

2.7 AMF and Disease Interaction

Plant diseases and pathogen attacks infect and reduce plant yield to a great extent. Plant diseases, especially soil borne diseases, are the most difficult to manage and excessive use of pesticides may pose threat to human health (Johansson et al., 2004). The AMF are the major components of the rhizosphere and play an important role in decreasing plant disease incidence. There have been reports showing that a minimum colonization level before pathogen infection is required for mycorrhiza to be effective in controlling disease incidence, suggesting that AMF suppressing P fertilization level may reduce the beneficial effect of AMF on disease incidence. For example, Bartschi et al. (1981) observed that mycorrhizal colonization protected *Chamaecyparis lawsoniana* roots against *Phytophthora cinnamomi* when it was well established before pathogen infection. In another study, Slezacek et al. (2000) showed that bioprotection of *Pisum sativum* roots against *Aphanomyces euteiches* is related to mycorrhiza-related chitinolytic enzymes which appeared to depend on fully established mycorrhizal symbiosis. On the other hand, studies have reported that a minimum threshold AMF colonization level is not necessary for mycorrhizal-induced disease reduction (Caron et al., 1986; St-Arnaud et al., 1994, 1997).

Mycorrhizal fungi protect plants against pathogen attack (Elsen et al., 2003; Hol and Cook, 2005). Some authors have reported that AMF biocontrol effect varied, depending on the plant species, nematode species and the AMF species involved

(Roncadori, 1997). Some AMF species, such as *G. mosseae*, are particularly effective in protecting plants against parasitic nematodes (Oyekanmi et al., 2008). Pathogen suppression has been ascribed to both physiological changes in mycorrhizal plants and direct interactions between AMF and pathogens. However, the precise mechanisms for protection are difficult to disentangle (Azcón-Aguilar and Barea, 1997).

Various mechanisms that could be involved in enhanced plant defence against pathogens have been proposed. According to Graham and Menge (1982), increased P content in AMF roots caused a reduction in root exudates thus reducing the activity of the pathogens. It has also been proposed that both AMF and root pathogens compete for root C. The higher demand of C by AMF may inhibit pathogen growth (Linderman, 1994). The AMF increase host plant tolerance to root pathogen attacks by compensating for the loss of root mass (Linderman, 1994) by nematodes (Pinochet et al., 1996) and fungi (Cordier et al., 1996). Pioneering observations indicated that AMF and root pathogens compete for same infection sites. For example, Cordier et al. (1996), showed that *Phytophthora* development is reduced in AMF-colonized and adjacent colonized regions of AMF root system, and that the pathogen does not penetrate arbuscule containing cells.

Changes in soil microbial populations induced by AMF formation may lead to stimulation of certain components of the microbiota, which in turn may be antagonistic to root pathogens (Linderman, 1994). The activation of plant defence mechanism as a response to AMF colonization is an important protective capacity of AMF. In general, various compounds involved in plant defence mechanisms studies in relation to AMF are phytoalexins, enzymes of the phenylpropanoid pathway, chitinases, β -1,3-glucanases,

peroxidases, pathogenesis-related proteins and phenolics (Gianinazzi-Pearson et al., 1994).

2.8 AMF Biomass and Fatty Acids

Mycorrhizal fungi form extensive ERM, which influence other soil microorganisms by bringing C to soil, enhance plant nutrition, and stabilize soil aggregates (Bingham and Biondini, 2009). The estimation of their biomass is thus important to understand their possible role in soil processes. Quantification of fungal hyphae by microscopy includes both living and dead hyphae, unless specialized vital staining methods are applied (Soderstrom, 1977; Sylvia, 1988) and may be biased by the presence of other fungi. One alternative to these methods involves the analysis of signature lipid biomarkers to study the abundance of AMF (Balser et al., 2005) and the structural diversity of microbial populations. Some fatty acids abundant in AMF are; 16:1 ω 5, 18:1 ω 7, 18:1 ω 9, 20:3, 20:4 and 20:5 (Graham et al., 1995; Jansa et al., 1999). Sakamoto et al. (2004) reported the presence of more abundant phospholipids fatty acid (PLFA) 16:1 ω 5 in sand inoculated with AMF than in non-inoculated sand. They reported that PLFA 20:1 ω 9 seems specific for *Gigaspora* species since it is closely correlated with amount of *Gi. rosea* biomass, and could be used as specific marker for identifying and quantifying the external hyphae of *Gi. rosea*. Madan et al. (2002) have successively used C16: 1 ω 5, C18:1 ω 9, C20:1 ω 9 as an indicator of AMF in soil. Balser et al. (2005) used fatty acid C16: 1 ω 5 and C18:2 ω 6 to detect and quantify AMF biomass in Hawaiian soils and concluded that phospholipids may be a valuable tool to study the abundance and interactions of AMF and other fungi in the field.

Fatty acid methyl ester (FAME) profiles have been used as biochemical characters to study many different groups of soil microorganisms (Tunlid and White, 1992; Zelles, 1999). The analysis of microbial membrane lipid components, specifically PLFAs, is an effective tool to measure the viable microbial biomass present in soil. In AMF, energy is mainly stored in neutral lipids (Olsson, 1999).

2.9 Arbuscular Mycorrhizal Fungi and Organic Matter Mineralization

Soil organic matter is a store house of nutrients. The nutrients present in organic combinations are released into soil solution through mineralization of organic matter by microorganisms. The nutrients absorbed by plants from the soil are stored in above-and below-ground biomass. When dead plants and animals enter soil, they are again broken down by various soil microorganisms, which use them as substrates for energy and also as nutrients sources in the synthesis of new cells, and the nutrients are again released into soil solution and the cycle continues. Most N in soil is, however, present in organic matter and plants rely on microorganisms to mineralize N from organic complexes. The role of AMF in plant acquisition of organic N is not clear. In view of the general perception that AMF lack the saprotrophic capability to enable N mineralisation, and are dependent on host plants for their C source, studies have not been directed on these lines. However, AMF has been reported to proliferate in organic matter and scavenge the mineral N released from soil organic matter (St. John et al., 1983; Hamel, 2004). AMF have been shown to develop arbuscules and vesicles in organic matter (Aristizábal et al., 2004) but their role in organic residue decomposition and mineralization is unclear. The hyphae of AMF can also take up amino acids (Hawkins et al., 2000; Gobindarajulu et al. 2005; Jin et al., 2005). Hodge et al. (2001) have used compartmented microcosm and ^{13}C

and ^{15}N labelled leaf material, and found that AMF enhances organic matter mineralization and increases N uptake by plants. In yet another compartmentalized experiment, Hodge (2003) showed that AMF can improve N recovery from organic patches in soil. Though there is no conclusive evidence of saprophytism in AMF, it seems that AMF indirectly influence organic matter decomposition by changing the soil microbial community (Wu et al., 2008). Arbuscular mycorrhizal fungi provide extensive conduits for allocation of C to soil system (Johnson et al., 2001) that could enhance microbial growth (Andrade et al., 1998). Some studies also report that extraradical mycelium of AMF excrete cellulase, pectinase and xyloglucanase hydrolytic enzymes (Garcia-Garrido et al., 1992, 2000) that may be involved in organic matter decomposition. The role of AMF in soil organic matter mineralization is still unclear and needs further study.

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3. Preface

The following chapter was published in *Mycorrhiza* (Atul-Nayyar, A., C. Hamel, K. Hanson, and J. Germida. 2009. The arbuscular mycorrhizal symbiosis links N mineralization to plant demand. *Mycorrhiza* 19: 239-246). The work reported here is a demonstration of the importance of AMF in nutrient cycling in soil. The study reports the new pathway of influence of AMF on N-cycling in plant-soil ecosystem. On this basis, it is important to recognize better the impact of cropping practices and soil fertility management on these fungi.

C. Hamel and J. Germida co-supervised this work. K. Hanson provided assistance with inoculum production. All co-authors reviewed the manuscript. I planned the experiment, processed and analysed samples, submitted samples for ^{15}N determination at AAFC, Lethbridge Research Station. I interpreted the data and prepared the manuscript for publication.

3. THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS LINKS N-MINERALIZATION TO PLANT DEMAND

3.1 Abstract

Arbuscular mycorrhizal fungi (AMF) facilitate inorganic N (NH_4^+ or NO_3^-) uptake by plants but their role in N mobilization from organic sources is still unclear. We hypothesized that arbuscular mycorrhizae enhance the ability of a plant to use organic residues as a source of N. This was tested under controlled glasshouse conditions by burying organic residue (OR) in soil separated by a 20- μm nylon mesh so that only fungal hyphae can pass through it. The fate of the N contained in the OR as influenced by *Glomus claroideum*, *G. clarum* or *G. intraradices* over 24 weeks, was determined using ^{15}N as a tracer. The AMF species enhanced N-mineralization from OR to different levels. N-recovery and translocation to Russian wildrye (*Psathyrostachys juncea* Fisch. Nevski) by hyphae reached 25% of mineralized N in *G. clarum*, which was most effective despite its smaller extraradical development in soil as compared to other AMF species. Mobilization of N by *G. clarum* relieved plant N deficiency and enhanced plant growth. We show that AMF hyphae modify soil functioning by linking plant growth to N-mineralization from OR. AMF species enhance N-mineralization differentially leading to species specific changes in the quality of the soil environment (soil C-to-N ratio) and structure of the soil microbial community.

3.2 Introduction

Nitrogen (N) availability often limits plant growth. A large amount of N is stored in soil organic matter, but plants generally absorb N as NH_4^+ and NO_3^- , the inorganic forms of N in soil, relying on microorganisms to mineralize N from organic forms. Some plants have developed the ability to directly utilize some simple soluble organic N compounds from soil (Jones and Darrah, 1992; Chapin et al., 1993) while others rely on specialized symbiotic associations to exploit organic N sources (Read, 1991). While the metabolism of ectomycorrhizal and ericoid mycorrhizal fungi gives their host plant (Abuzinadah and Read, 1989; Leake and Read, 1990) the ability to use organic matter as a source of N in heath lands and forests, the role of arbuscular mycorrhizae in plant acquisition of organic N is unclear.

The AMF are known as obligate biotrophs relying on C provided by their host plant rather than on dead organic matter (Nakano et al., 1999; Sawers et al., 2008). Most of the literature related to the role of arbuscular mycorrhiza in plant growth and nutrition has been related to uptake of immobile ions of plant nutrients, such as orthophosphate (Li et al., 2006). The AMF hyphal network is important in giving plants access to low mobility ions located far from the root surface. Studies using ^{15}N tracer techniques have shown that AMF hyphae can transport from soil to roots (Johansen et al., 1994; Mader et al., 2000; Tanaka and Yano, 2005; Jackson et al., 2008), but high mobility and rapid movement of mineral N to roots through mass flow (Tinker and Nye, 2000) has suggested that AMF play little role in plant N nutrition. The AMF symbiosis was traditionally considered unimportant in plant N nutrition (Liu et al., 2007).

The AMF have been reported to proliferate in organic matter and scavenge the mineral N released from soil organic particles (St. John et al., 1983; Hamel, 2004). The hyphae of

AMF can also take up amino acids (Hawkins et al., 2000; Govindarajulu et al., 2005). Based on the observation of hyphae and vesicles of AMF in decomposing leaves of *Myrica parvifolia*, *M. pubescens* and *Paepalanthus* sp., Aristizábal and collaborators (2004) proposed that AMF enter decomposing leaves through vascular tissues and efficiently recycle the mineral nutrients released by microbial decomposers. The ability of AMF to use dead organic substrates (Talbot et al., 2008) is a matter of debate, but even if this ability is small or nonexistent, these fungi may be important in N cycling through their influence on the free-living soil microbial community (Andrade et al., 1997; Marschner et al., 2001; Hodge, 2003b; Aneja et al., 2006), which is responsible for most of the N-mineralization, particularly in grassland ecosystems (Stanton, 1988). Arbuscular mycorrhizal fungi form symbiotic associations with most land plant species (Newsham et al., 1995) and could importantly influence N-recycling from litter and soil organic matter. Hodge et al. (2001) reported that *Glomus hoi*, an AMF, enhances organic matter decomposition with no effect on plant growth, but their experiment was of short duration (42 days) and restricted to one AMF. As plant demand increases with time, we expect that study of longer duration may reveal better the availability of N to plant from decomposing organic matter. We also hypothesized that different AMF species may have different influence on organic matter mineralization.

The objective of this study was to demonstrate the key role of AMF symbiosis in linking the process of N-mineralization to plant N-demand in soil, where the AMF symbiosis regulates the recycling of plant residue N into living plant biomass and in the process, changes the soil environment. We used the perennial grass Russian wildrye (*Psathyrostachys juncea* Fisch. Nevski) and three AMF species in a controlled condition

experiment where extraradical AMF hyphae were allowed to access a patch of ^{15}N -labelled organic residues contained in nylon mesh, which we buried in soil. We examined the effect of arbuscular mycorrhizae on residue decomposition, as compared to a non-mycorrhizal control, and the consequences of this effect on plant growth, the soil environment, and microbial community structure, after 24 weeks.

3.3 Materials and Methods

3.3.1 Plants and AMF material

Seeds of Russian wildrye (cultivar Swift) were sown in pots containing 500 g of pasteurised soil (80°C for 3 h). The Brown Chernozemic soil was loamy sand with pH of 6.5, EC of 0.48 mS and contained 19.7 μg of $\text{NH}_4\text{-N}$, 14.1 μg of $\text{NO}_3\text{-N}$, 21.3 μg of $\text{PO}_4\text{-P}$ and 324.5 μg of K g^{-1} soil after pasteurization. The soil was taken from a cultivated field located about 25 km Northwest of Swift Current, Saskatchewan. Pots were inoculated with one of three different AMF species or with sterilized inoculant. All pots also received 2 mL of a filtrate of the three AMF inoculum mixed together in equal proportion and filtered through Whatman No. 1 to provide inoculum specific microbial population to control systems. Each mycorrhizal treatment received 1g of root inoculum thoroughly mixed with the soil. The AMF species used were *Glomus intraradices* (Schenck & Smith DAOM 181602), *Glomus claroideum* (Schenck & Smith DAOM 235379) and *Glomus clarum* (Nicolson & Smith DAOM 235378). All AMF species were multiplied from spores using corn (*Zea mays* L. var. Sunnyvee) grown for 60 d in a greenhouse. At harvest roots were washed, air dried, chopped and stored at 4°C until use. Control plants received 1 g of autoclaved inoculum. Plants were maintained at a

day/night temperature of 22°C/18°C with a 16-h photoperiod in a growth cabinet before transplanting to the experimental pots after 21 d.

3.3.2 Experimental design

Four Russian wildrye seedlings colonized by one of the three AMF species or non-mycorrhizal were transplanted in pots. A patch of ^{15}N -labelled organic residues was inserted in each pot at the time of transplanting. The organic residue (OR) patch was made of 4 g of ^{15}N -labelled root and shoot of wheat plants. Wheat was labelled with $^{15}\text{NH}_4^{15}\text{NO}_3$, 99% atom and grown for 45 days. After 45 days, wheat plants were harvested, dried and ground (<2 mm). The ground labelled material was mixed with some pasteurised soil and inserted in pots. The organic material contained 22 mg N, 38% of which was ^{15}N . The patch material was sandwiched between two 20- μm nylon mesh walls held onto a PVC ring. Walls were a wire mesh covered by the nylon mesh (Nitex Bolting Cloth; Wildco, Buffalo, NY) on both sides creating an air gap preventing N diffusion. The patch was placed in the root zone with mesh facing towards the centre of the pot. The small patch volume (0.05 L) as compared to pot volume (6 L) meant that the soil volume available to mycorrhizal and non-mycorrhizal plants was practically the same. Pots were filled with the pasteurized loamy sand. Nitrogen (315 mg $\text{NH}_4\text{SO}_4 \text{ pot}^{-1}$) was mixed in the soil at the start of the experiment, and 15 mL of a modified Long Ashton nutrient solution containing in 554 mg L^{-1} KCl, 200 mg L^{-1} $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 244 mg L^{-1} MgSO_4 , 520 mg L^{-1} $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 1.7 mg L^{-1} MnSO_4 , 0.25 mg L^{-1} $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.30 mg L^{-1} $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.0 mg L^{-1} H_3O_3 , 5.0 mg L^{-1} NaCl, 0.09 mg L^{-1} $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, and 32.9 mg L^{-1} NaFe-EDTA was added at transplantation.

Treatments were replicated five times and pots were arranged in a randomized complete block design in the greenhouse. Plants were also maintained at day/night temperature of 22°C/18°C with a photoperiod of 16h under mixed natural and supplemental light from Lumalux high pressure sodium lamps (Osram Sylvania, Mississauga), and plants were watered every three days. Saucers were used and watering was done parsimoniously to prevent all N loss during the experiment. Plants were grown for a period of 24 wks between 22 February and 26 July 2007.

3.3.3 Sampling and analysis

At harvest, plant shoots were cut at ground level and their biomass was determined after drying at 40°C to constant weight. Roots were recovered on a 2-mm sieve and thoroughly washed with tap water to remove adhering soil. Soil and OR patch materials were sieved through 2 mm before subsampling for further analysis. The OR patch material was dried at 40°C and weighed. Roots were cut into 1-cm fragments, weighed fresh, and subsampled. One subsample was dried at 40°C until constant weight for moisture content determination and total root dry mass calculation, and for plant tissue N and P analyses. Ground shoot and root tissue digestion (Varley, 1966) was completed and tissue N and P concentrations were measured by the salicylate/nitroprusside (Noel and Hambleton, 1976), and the acidic molybdate/ascorbic acid method a (Milbury et al., 1970), respectively, on a Technicon AutoAnalyzer II. The second root subsample was used for determination of AMF root colonization using the gridline intersect method (Giovannetti and Mosse, 1980). Roots were cleared in boiling 10% KOH solution for 10 min, rinsed with tap water, and stained for 3 min in boiling ink-vinegar solution (Vierheilig et al., 1998).

Shoots and roots of Russian wildrye, bulk soil, and OR patch material were ground to a fine powder in a ball mill. The ground samples were analysed for ^{15}N content by NCA analyzer Carlo Erba NA1500 coupled to a mass spectrometer Optima (Cheshire, United Kingdom). Total C and N in OR patch material was analyzed using Carlo Erba NA1500 NCS analyzer. The amount of N from organic residues remaining in OR patches (N_{ORr}) at harvest were calculated as:

$$N_{\text{ORr}} = (^{15}\text{N}_{\text{tot}} \cdot N_{\text{tot}} - 0.3663 \cdot N_{\text{tot}}) / (^{15}\text{N}_{\text{ORi}} - 0.3663) \quad [3.1]$$

where “ N_{tot} ” is the amount of N in the OR patch at harvest, “ $^{15}\text{N}_{\text{tot}}$ ” is the percentage of ^{15}N measured in the OR patch at harvest, “0.3663” is the percentage of ^{15}N in the soil initially placed in the patch, and “ $^{15}\text{N}_{\text{ORi}}$ ” is the percentage of ^{15}N in the plant residues initially placed in the patch.

The amounts of N mineralized from OR patches were calculated as the difference between N amounts placed in OR at the start of the experiment and N amounts at the end of the experiment (N_{ORr}). The percentage of mineralized N recovered by plants was calculated as:

$$\% \text{ recovery} = 100 \cdot (^{15}\text{N}_s \cdot N_s + ^{15}\text{N}_r \cdot N_r) / \text{N mineralized from OR patch} \quad [3.2]$$

where $^{15}\text{N}_s$ and $^{15}\text{N}_r$ are the percentages of ^{15}N measured in shoot and root, and N_s and N_r are the total N content in shoot and root, respectively.

We sought inoculation treatment effects on the structure of the soil microbial community active in the soil with decomposing OR through comparison of their phospholipid fatty acids (PLFA) profiles. Phospholipid fatty acids were analyzed as described previously (Hamel et al., 2006). Briefly, soil lipids were extracted from 4 g of soil in dichloromethane (DMC): methanol (MeOH): citrate buffer (1:2:0.8 v/v). Lipid-

class separation was conducted in silica gel columns. Fatty acid methyl esters from the phospholipid fraction were created through mild acid methanolysis. Fatty acid methyl esters dissolved in hexane were analyzed using a Varian 3900 gas chromatograph (GC) equipped with a CP-8400 auto sampler and a flame ionization detector (FID). Methyl nonadecanoate (19:0, Sigma Aldrich) added to samples served as an internal standard for the quantification of FAME. Helium was the carrier gas (30 ml min⁻¹) and the column was a 50-m Varian Capillary Select FAME # cp7420. Peak identification was based on comparison of retention times to known standards (Bacterial Acid Methyl Esters #47080-U, Supelco, Bellefonte, USA). The PLFAs i-15:0, a-15:0, i-16:0, i-17:0, were used as biomarkers for Gram positive bacteria (Sundh et al., 1997), PLFAs 3OH-14:0 for Gram negative bacteria (Spring et al., 2000), PLFA 18:1 ω 9t, 17:0, 15:0, 2OH-14:0, and 2OH-16:0 as general bacteria biomarkers (Kawashima et al., 1996; Sundh et al., 1997; Spring et al., 2000), and PLFAs 18:2 ω 6c and 18:1 ω 9c for fungi (Petersen and Klug, 1994; Sundh et al., 1997). The fatty acid nomenclature follows the omega form, A:B ω C, where A designates the number of carbon atoms, B the number of double bonds, and C the distance of the closest unsaturation from the aliphatic end of the molecule. Prefixes i- and a- refer to iso and anteiso methyl-branching. The suffixes c and t indicate *cis* and *trans* geometric isomers. Hydroxy groups are indicated by OH.

Hyphal length density (HLD) in bulk soil was measured using the line intersect method (Newman, 1966). Hyphae were extracted using a flotation–centrifugation technique (Dalpé and Hamel, 2008). Hyphae were stained for 5 min with trypan blue (Koske and Gemma, 2000) in the filtration unit. The stain was washed with distilled

water and hyphae were counted under a dissecting microscope. Hyphal lengths were calculated using the following formula.

$$\text{HLD} = \pi \text{NA} / 2\text{H} \quad [3.3]$$

where “N” is number of intersects between hyphae and the nitrocellulose filter gridlines, “A” is the surface area of filter and “H” is the total length of lines. Hyphal length densities for each treatment were calculated from hyphal lengths and expressed as mm g^{-1} soil. Background values from uninoculated control were subtracted and values above control were considered AMF hyphal length densities (HLD_{AMF}).

3.3.4 Statistical analysis

The significance of treatment effects on soil and plant variables were assessed by ANOVA using JMP 3.2.6, (SAS Institute, Cary, USA) and means were compared using Fisher’s LSD test at $\alpha = 0.05$. Differences in soil microbial community composition was determined by discriminant analysis (Huberty, 1994) using normalized and standardized PLFA biomarker values using the backward stepwise procedure in Systat v. 12 (Point Richmond, USA). At each step, the biomarker with the least F-to-remove value was removed from the model, until no biomarker with value below 0.15 remained. Regression analysis was conducted using JMP 3.2.6 to relate the level of AMF root colonization and mineralized N recovery by plants.

3.4 Results

3.4.1 Mycorrhizal development

All three AMF spp. produced AMF colonization levels ranging from 18.8% to 25.6% in the roots of Russian wildrye (Table 3.1), levels similar to those that can be observed in the field in our area. Uninoculated plants showed no colonization at harvest

Table 3.1 Amount of N mineralized from organic residue (OR) over the 24-week growth period, final C-to-N ratio of the organic residue patch material, Russian wildrye shoot dry weight (DW), root DW, N concentration in shoot and root, and arbuscular mycorrhizal (AMF) root colonization, and AMF hyphal length density (HLD) in rooting soil at harvest, as influenced by inoculation treatments.

Inoculation treatments	Control	<i>G. claroideum</i>	<i>G. clarum</i>	<i>G. intraradices</i>
N mineralized from residues (mg)	1.41 c	2.91 ab	4.02 a	2.73 b
OR Patch materials C-to-N ratio	12.3 a	9.8 b	9.6 b	9.8 b
Shoot DW (g)	10.7 b	10.7 b	12.4 a	13.2 a
Root DW (g)	9.9 b	10.2 b	14.4 a	13.3 a
Shoot N (mg g ⁻¹)	15.9 b	19.0 b	33.5 a	16.2 b
Root N (mg g ⁻¹)	12.6 a	13.2 a	11.5 a	12.3 a
Shoot P (mg g ⁻¹)	2.1 a	2.2 a	2.0 a	2.1 a
Root P (mg g ⁻¹)	1.7 a	1.4 a	1.5 a	1.5 a
AMF colonization (%)	0	18.8 b	25.6 a	21.3 ab
AMF fungi HLD (mm g ⁻¹ soil)	0	6.17 a	5.35 b	6.74 a

Means ($n = 5$) followed by different letters in the same row are significantly different at $P < 0.05$ according to Fisher's protected LSD test.

(Table 3.1). Colonization was highest in *G. clarum* inoculated plants while *G. claroideum* colonized Russian wildrye to a lesser extent. Conversely, *G. clarum* had lower HLD_{AMF} than *G. claroideum* and *G. intraradices* in rooting soil (Table 3.1).

3.4.2 Mineralization of organic residue and microbial community in OR patches

The carbon-to-nitrogen ratio in the OR patch material had decreased from an initial level of 17.8 to less than 13, at harvest, and was lower in presence of AMF (Table 3.1) indicating that organic matter decomposition proceeded further in AMF than non-mycorrhizal systems. Accordingly, we found larger ($P = 0.01$) amounts of mineralized N in presence of AMF than in control systems (Table 3.1).

The structure of the microbial communities colonizing the decomposing patch material under differed inoculation treatments was distinct (Fig. 3.1). Discriminant analysis classified 100% all microbial communities into their respective inoculation treatment, based on their PLFA profiles (Wilk's lambda = 0.003, $P = 0.004$), indicating that inoculation treatments had shaped microbial communities. Only one soil microbial community associated with *G. intraradices* was misclassified as belonging to a *G. clarum* colonized soil. Variation in microbial community structure in OR patches were due to modifications in bacterial and fungal saprotrophic populations. Six bacterial biomarkers (15:0, i-16:0, i-17:0, 17:0, 2OH-14:0, 2OH-16:0) and three fungal saprotroph biomarkers (18:1 ω 9c, 18:2 ω 6c, and 18:1 ω 9t) were retained as the explicative variables, in the model. Inoculation treatments induced qualitative changes in the structure of microbial communities in the OR patch material, but did not significantly influence total microbial biomass, as estimated by the sum of microbial PLFA biomarkers in lipid extracts (Appendix A.1).

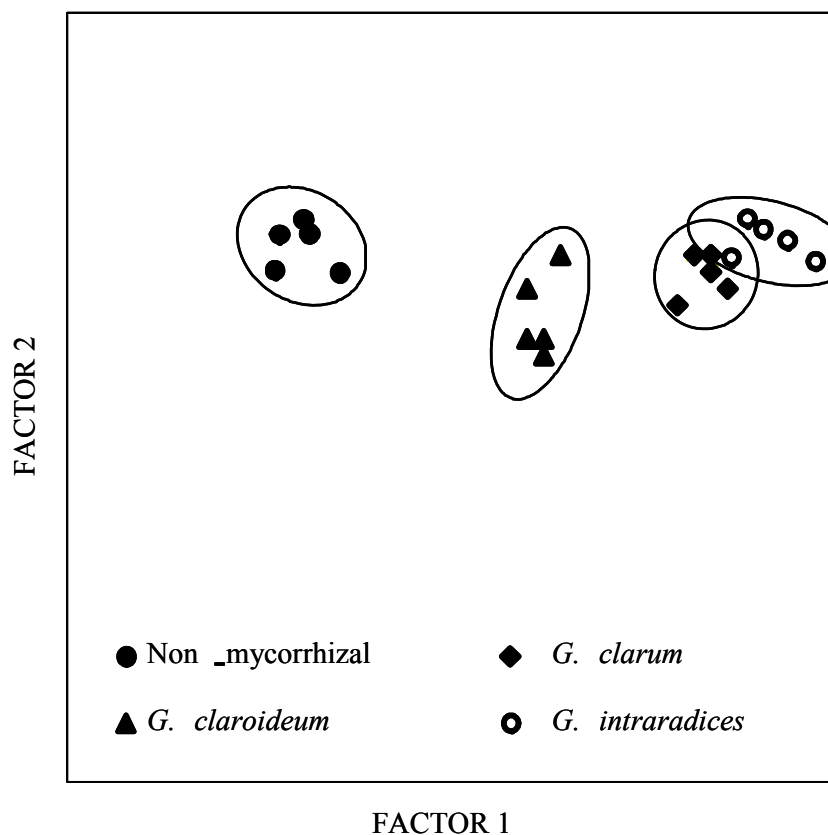


Figure 3.1. Biplots from a discriminant analysis showing the effect of inoculation treatments on the microbial community structure in organic residue patches after 24 weeks. Microbial communities in organic residue patches can correctly be classified (circles) into their respective inoculation treatments based on PLFA biomarker profile (Wilk's lambda = 0.003, $P = 0.004$).

3.4.3 Plant biomass and nutrition

Shoot and root dry mass varied with inoculation treatments. Russian wildrye in symbiosis with *G. clarum* and *G. intraradices* produced 16% and 23% more shoot biomass than non-mycorrhizal control plants, while average shoot biomass of *G. claroides* colonized plants did not significantly differ from control (Table 3.1). Root dry mass of *G. clarum* and *G. intraradices* inoculated plants was 45% and 34% larger than that of control plants (Table 3.1). Russian wildrye shoot N-concentration was increased by *G. clarum*, but no inoculation effects were found on the concentration of P in shoot ($P = 0.56$) or root ($P = 0.20$) (Table 3.1). This indicates that plants were P sufficient, but N limited, a limitation which was relieved by *G. clarum*. Plants colonized by three mycorrhizal species were effective in capturing the N mineralized in the OR patch as compared to control (Fig. 3.2). A strong relationship ($R^2 = 0.80$, $P < 0.001$, $N = 20$) between mineralized N recovery by plants and AMF root colonization level suggests that extent of root colonization is important in determining N-mineralization and transfer by AMF to plants.

3.5 Discussion

We verified that AMF can increase the mobilization and enhance plant N uptake N from decomposing organic residues, and found that this effect can be important in N cycling. The presence of AMF hyphae in OR patches increased N-mineralization by 228%, on average. Up to 25% of mineralized N was recovered by AMF Russian wildrye plants, after 24 weeks, and this proportion would have probably been larger if mineralized N diffusion from the patch to plant roots was not minimized by the double mesh walls of OR patch containers. Hodge et al. (2001) measured about 15% of patch N

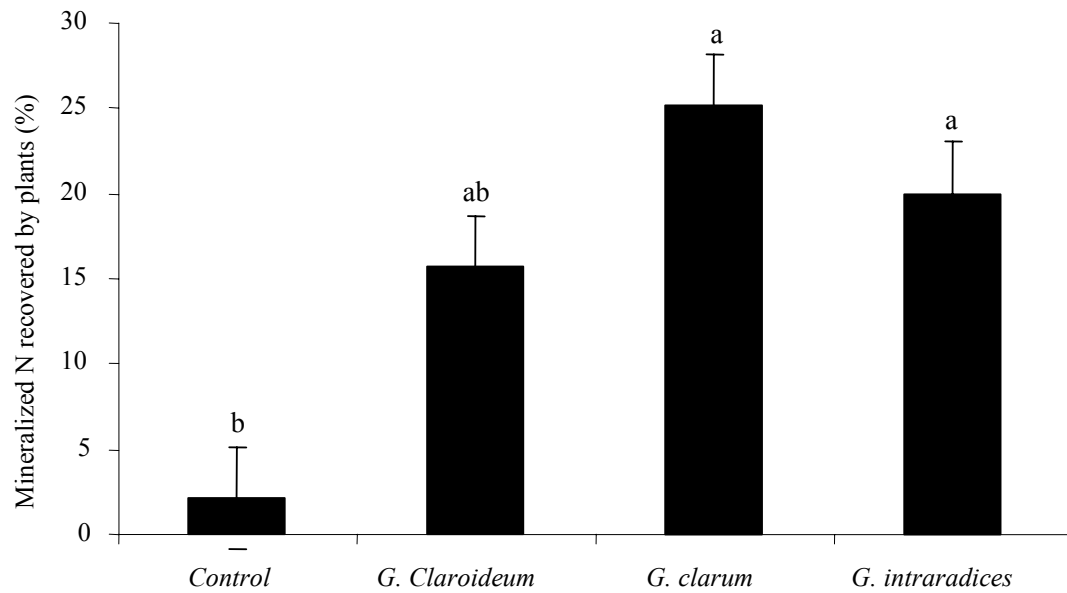


Figure 3.2. Proportion of the N released through mineralization of organic residues that was recovered by Russian wildrye plants inoculated with different AMF species or uninoculated control. Different letters indicate different means (Fishers LSD, $P = 0.02$, $n = 5$). Bars are standard errors.

recovery in mycorrhizal *Plantago lanceolata* in an experiment that lasted only 42 days. It appears that plants can stimulate the mineralization of OR in soil through C investment in AMF development. Observations of enhanced N-mineralization and plant recovery of mineralized N reveal a role for AMF in the regulation of efficient N cycling in soil, where these fungi link plant N needs to N-mineralization. The data show that in absence of AMF hyphae, OR mineralization proceeds slowly, reducing the risk of N loss from the system since NO_3^- is susceptible to denitrification and leaching losses. It appears that stimulation of N-mineralization by AMF hyphae may be regulated by plant N demand, as high soil N availability reduces AMF extraradical development (Liu et al., 2000), which has been related to plant uptake of mineralized N (Hodge, 2003b; Hodge et al., 2001).

We can attribute plant growth enhancement in *G. clarum* inoculated systems to improved N nutrition. Increased plant tissue N concentration and absence of effect on tissue P concentration clearly indicate that *G. clarum* relieved N deficiency in its host. The cause of improved plant growth with *G. intraradices* is less clear, as the concentration of nutrients in plant tissues were not different from that of control plants. Finally, inoculation with *G. claroideum* did not influence plant biomass production. Although all AMF could enhance N mineralization from the OR patch, this effect was not always associated with improved plant productivity, showing difference in functionality among the AMF used.

We found that the extent of extraradical development might not be the only factor involved in AMF-induced N-mineralization enhancement. *G. clarum* seemingly produced less extraradical hyphae in the bulk soil but enhanced most N-mineralization. The fact that the growth of fungus may have been preferentially stimulated in the OR

patch cannot be ruled out, however. *G. clarum* enhanced transport of N to plant, and plant biomass production despite it having the lowest HLD_{AMF} development. Better plant growth performance with *G. clarum* was associated with higher AMF root colonization, however, suggesting that a bottleneck at the plant-fungus interface might be a factor limiting effective N-transfer from decomposing organic matter to plants via AMF hyphae.

The faster decomposition of organic residues in OR patches in presence of AMF hyphae could be due to direct or indirect effects. Direct effects of arbuscular mycorrhizae on organic residue decomposition could be due to enzymatic decomposition by extraradical hyphae. The mycelium of AMF proliferates in organic residue (Ravnskov et al., 1999) and has the ability to excrete hydrolytic enzymes (Varma, 1999). Various hydrolytic enzymes such as cellulase, pectinase and xyloglucanase have been reported in external mycelium of AMF (Garcia Romera et al., 1991; Garcia-Garrido et al., 1992). These enzymes are known to be involved in the degradation of plant material in soil. The AMF can arguably be involved directly in the mineralization of organic residues (Talbot et al., 2008), at least to some extent.

The AMF may also stimulate OR decomposition through their effect on soil microorganisms. Soil microbial growth can be stimulated (Secilia and Bagyaraj, 1987; Andrade, 2004), and the soil microbial community changed (Posta et al., 1994; Marschner and Crowley, 1996a; Marschner and Crowley, 1996b) in the presence of AMF hyphae (Andrade et al., 1997; Andrade, 2004). We found no difference in active soil microbial biomass between treatments at harvest, but it could have been larger in AMF colonized systems initially and declined with narrowing patch material C-to-N ratio, as

OR mineralization proceeded. Qualitative changes in microbial community structure may also be responsible for faster organic matter decomposition in the presence of arbuscular mycorrhizae. The AMF may influence the soil microbial community through different mechanisms including modification in plant signalling or defence-related biochemical pathways (Lioussanne et al., 2008) and the modification of the nature, amount and distribution of plant-derived C compounds in soil (Toljander et al., 2007). Extraradical hyphae of AMF may bring available C to microorganisms of the hyphosphere allowing them to mineralize recalcitrant soil organic matter, as described in the model of Schimel & Weintraub (2003). In absence of AMF effect on the abundance of microbial PLFA biomarkers, Hodge et al. (2001) concluded to the probable direct involvement of a *G. hoi* strain in N mineralization from OR. However, a biomass similar in size but more active could have enhanced mineralization in that study. Also, hyphospheric effects could have been diluted and masked by a large soil volume in the OR compartment used. The observation of better N uptake in a non-host plant species in soil with AMF (Hodge, 2003a) indicates that, directly or not, these fungi can enhance substantially N-cycling in soil.

We observed differences in the structure of soil microbial communities in OR patches after 24 weeks. These differences may reflect both the influence of arbuscular mycorrhizae and that of organic materials at different stages of decomposition under different treatments. Change in the soil microbial community with decomposing organic residue was reported in other studies (Aneja et al., 2006; Ha et al., 2008). Decrease in the abundance of easily metabolized compounds with time drives a microbial succession for decomposing residues. Different carbon-to-nitrogen ratios in the OR patch material under

different treatments indicates that OR mineralization had proceeded further in presence of AMF hyphae and thus, that different qualities of soil organic matter could select soil microbial communities with different structure.

Results suggest new pathways of influence by arbuscular mycorrhizae on plant-soil ecosystems. We have shown that arbuscular mycorrhizae link plant N needs and growth to OR mineralization, modifying the abundance of soil resources and impacting the structure of the soil microbial community.

3.6 Literature Cited

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4. Preface

This work has been published in *Applied Soil Ecology* (Atul-Nayyar, C. Hamel, T. Forge, F. Selles, P. G. Jefferson, K. Hanson, J. Germida. 2008. Arbuscular mycorrhizal fungi and nematodes are involved in negative feedback on a dual culture of alfalfa and Russian wildrye. *Appl. Soil Ecol.* 40: 30-36). This work revealed the interactive effect of P fertilization and cropping system on AMF. The unexpected persistence of alfalfa in mixture with RWR in a long-term experiment suggested negative interactions between plants and AMF influencing crop performance in mixture. The work was co-supervised by C. Hamel and J. Germida. F. Selles and P. Jefferson initiated and maintained this field experiment and T. Forge evaluated nematode population. Co-authors helped in editing the manuscript. I prepared a research proposal, plan sampling, lead the sampling team, processed and analysed most samples, coordinated analytical work done in AAFC service chemistry laboratory and prepared the manuscript.

4. ARBUSCULAR MYCORRHIZAL FUNGI AND NEMATODES ARE INVOLVED IN NEGATIVE FEEDBACK ON A DUAL CULTURE OF ALFALFA AND RUSSIAN WILDRIE

4.1 Abstract

The complex biological interactions taking place in soil-plant systems may sometimes alter the functioning of an ecosystem. We examined the relationship between AMF root colonization, nematode populations, and plant competition in an eight-year old field experiment comparing alfalfa monoculture to dual culture of alfalfa (*Medicago sativa* L.) and Russian wildrye (*Psathyrostachys juncea* Fisch. Nevski), (RWR), grown under different soil P fertility levels, in a Brown Chernozemic soil in Saskatchewan. The experiment included three P rates: 0, 20 and 40 kg P₂O₅ ha⁻¹ (0P, 20P, 40P) applied annually and was sampled three times during the cropping season: June 30, September 1 and September 29, 2004. Higher AMF symbiotic development compensated for reduced soil P fertility in alfalfa stands without RWR and forage dry matter yield was not affected by P rates. In the presence of RWR, reduced soil P fertility at 0P and 20P led to a forage yield reduction. Fertilization P treatments modified the soil microbial community structure only in the presence of RWR, as revealed by discriminant analysis of the soil profiles of microbial phospholipids fatty acid indicators in soil lipid extract and functional nematode groups. Arbuscular mycorrhizal root colonization level was reduced with P fertilizer both in the presence and absence of RWR. In the presence of RWR, lower plant root colonization was concurrent with higher abundance of total, fungivorous and omnivorous nematodes. Our results are consistent with a model of negative feedback

from the plant-associated soil microflora where the presence of RWR increased the population of fungivorous nematodes and grazing of AMF hyphae. Negative impacts were larger in low P fertility soils promoting AMF symbiotic development. The unexpected decrease in alfalfa-RWR dual culture yield under 0P and 20P fertilization levels was attributed to a carbon drain created by enhanced nematode feeding on AMF in presence of RWR.

4.2 Introduction

Perennial forage crops are good for soils in addition to feeding sheep and cattle. They are seen as soil improving crops that add organic matter to the soil and serve as a permanent ground cover that prevents soil erosion. Planting legumes and grasses in forage stands is advantageous. It has long been known that mixtures of legumes and grasses provide greater forage yield than monocultures (Ledgard and Steele, 1992; Zemenchik et al., 2002). Legumes can fix atmospheric nitrogen through the activity of symbiotic bacteria that live in nodules on their roots. This N fixation provides N to both the legume and grass components of mixed stands (Ledgard and Steele, 1992). Furthermore, since legumes have higher protein content than grasses, mixed stands have better protein content. Thus, the inclusion of alfalfa (*Medicago sativa* L.) in grass forage stands is known to improve forage and livestock production (Kreuger and Vigil, 1979). However, alfalfa plants are typically negatively impacted by associated grasses, which are better competitors for nutrients and water in semiarid regions (Kilcher and Heinrichs, 1966) such as southwest Saskatchewan. In particular, Russian wildrye (*Psathyrostachys juncea* Fisch. Nevski), a high-yielding forage grass grown in this region, is very competitive.

In a field experiment set up in 1997 at Swift Current Research station to define best agronomic practices for the maintenance of a high-yielding alfalfa-RWR dual culture, RWR was expected to out compete alfalfa over time. But in contrast, the proportion of alfalfa grown in mixture with RWR did not decline with years and, furthermore, lower yields of alfalfa-RWR dual culture than alfalfa monoculture were repeatedly obtained (Selles and Jefferson, 2004). Based on these results, we hypothesized that the unexpected interactions observed between alfalfa and RWR and the low productivity of the alfalfa-RWR dual culture were related to the influence of the associated soil microflora on these crop plants. The soil microbial community, soil environment, plant nutrition and productivity were described during the growing season to verify this hypothesis.

4.3 Materials and Methods

4.3.1 Study site and experimental design

The experiment was conducted on a Brown Chernozemic soil located at the South Farm of the Semiarid Prairie Agricultural Research Centre in Swift Current, Saskatchewan, Canada. The soil was loamy clay with 2.28% organic matter, slightly acidic with pH of 6.62 and EC 0.32 of mS cm^{-1} . The experiment was initiated in 1997, i.e., eight years before the present study. It was set up in a randomised complete block design replicated four times. There were two plant diversity treatments, alfalfa (*Medicago sativa* L.) monoculture (cv. Rangerlander) and dual culture of alfalfa and Russian wildrye (cultivar Swift Russian Wild Ryegrass). Different amounts of P: 0, 20, or 40 $\text{kg P}_2\text{O}_5 \text{ ha}^{-1}$ (0P, 20P and 40P), were applied as triple super phosphate annually to these stands. Fertilizer was band placed with double disk opener between rows at 2.5 cm depth. Plots

were 1.83m X 6m and were seeded initially with a plot seeder at the rate of 25 seeds 30 cm⁻¹ and at 30.5 cm width row spacing. Average monthly temperature and total precipitation was recorded for the year 2004 (Table A.2)

4.3.2 Soil and plant sampling

The experiment was sampled three times during the growing season: on June 30th, September 1st and September 30th 2004. Intact soil cores (5 cm diameter) were taken from between rows at two randomly selected locations between rows in the top 7.5 cm soil layer of each plot using a foot sampler. Roots were extracted from these cores and washed prior to processing for AMF colonization determination. The top 7.5 cm layer of the soil was sampled also with a bulb planter for lipid analysis. Five cores were taken from different locations in each plot and pooled in plastic bags. Samples were refrigerated until sieving through 2mm, and then frozen at -20⁰C until lipid extraction. Soil PO₄ flux was measured *in situ* using 6.5 x 2.25 cm anion exchange membranes (AEM) (#200253, Ionics, Watertown, Massachusetts). A set of three AEM was placed in each plot to a depth of 7.5 cm. The AEMs were inserted near the soil surface (2-7 cm) through the slit opened with a small shovel. These AEMs were inserted in the soil of each plot on May 17th, and were replaced on June 30th, and September 1st. Once removed from the soil, the membranes were rinsed with distilled water to remove the adhering soil particles and immediately placed in tubes containing 25 mL of 1 M KCl and brought to the laboratory for analysis as described in section 4.3.5.

Plant shoots were taken from two randomly selected 1-m row lengths in each plot, only on June 30th and September 1st, since there was no re-growth between September 1st and 30th. They were dried at 40⁰C until their weight remained constant, and total dry

matter was recorded. Soil samples using cores (2.5 cm diameter) were sampled from ten different locations and pooled as one sample for determining nematode population in each plot.

4.3.3 Fatty acid methyl ester (FAME) analysis

Fatty acids in the soil phospholipid (PLFAs) and neutral lipid (NLFAs) fractions were extracted and analyzed as a measure of active soil microbial biomass and storage material of fungal origin, respectively, using the method described in Hamel et al. (2006). Total soil lipids were extracted from 4 g of soil (dry weight equivalent) in dichloromethane (DCM): methanol (MeOH): citrate buffer (1:2:0.8 v/v). Lipid-class separation was conducted in silica gel columns. The neutral, glyco- and phospholipids fractions were eluted by sequential leaching with DCM, acetone and MeOH, respectively. The glycolipid fraction was discarded. The neutral and phospholipid fractions were dried under a flow of N₂ at 37°C in the fume hood, dissolved in two ml of MeOH for PLFA or DCM for NLFA and stored at -20°C. Fatty acid methyl esters were created through mild acid methanolysis. Ten µL of methyl nonadecanoate fatty acid (19:0; Sigma Aldrich) was added to serve as internal standard and samples were dried under a flow of N₂ at 37°C in the fume hood. Samples dissolved in 50 µL of hexane were analyzed using a Varian 3900 gas chromatograph (GC) equipped with a CP-8400 auto sampler and a flame ionization detector (FID). Helium was the carrier gas (30 mL min⁻¹) and the column was a 50-m Varian Capillary Select FAME # cp7420. Sample (2µL) injection was in 5:1 split mode. The injector was held at 250°C and the FID at 300°C. The initial oven temperature, 140°C, was held for 5 min, raised to 210°C at a rate of 2°C min⁻¹, then raised from 210°C to 250°C at a rate of 5°C min⁻¹, and held for 12 min. Identification of peaks was based on

comparison of retention times to known standards (Supelco Bacterial Acid Methyl Esters #47080-U, plus MJS Biolynx #MT1208 for 16:1 ω 5). The abundance of individual PLFAs was expressed as $\mu\text{g PLFA g}^{-1}$ dry soil. Amounts were derived from the relative area under specific peaks, as compared to the internal standard (19:0) peak value, which was calibrated according to a standard curve made from a range of concentrations of the 19:0 FAME standard dissolved in hexane. Fatty acids were named according to the ω -designation described as follows: total number of carbons followed by a colon; the number of double bonds; the symbol ω ; the position of the first double bond from the methyl end of the molecule. Cis and trans isomers are indicated with c or t, respectively. Methyl (meth) and hydroxy (OH) groups are labelled at the beginning, where applicable. Iso and anteiso forms are indicated by i- and a-, respectively.

Individual fatty acids have been used as signatures for various groups of microorganisms (Pankhurst et al., 2002; Hamel et al., 2006). The FAME 18:2 ω 6c and 18:1c were used as indicators of fungal biomass (Petersen and Klug, 1994; Frostegård and Bååth, 1996) and FAME 16:1 ω 5, as indicator of arbuscular mycorrhizal fungi (Spring et al., 2000; Balser et al., 2005). FAMES 3OH-12:0, a-12-meth-15:0, i-13-meth-15:0, 15:0, 2OH-14:0, i-14-meth-16:0, 16:1 ω 7c, i-15-meth-17:0, 17:0, 2OH-16:0, and 18:1t were chosen to represent bacterial PLFAs based on the bacterial standards used.

4.3.4 Percent root colonization

Roots were cut into 1 cm fragments and boiled in a 10% KOH solution for 10 min. Roots were then rinsed with tap water, stained by boiling for 3 min in ink-vinegar solution and rinsed with tap water acidified with a few drops of vinegar (Vierheilig et al., 1998). Root samples were kept in acidified water at 4°C until determination of their

percentage of AMF colonization by the gridline intersect method, under the dissecting microscope, as described by Giovannetti and Mosse (1980).

4.3.5 Soil PO₄ flux and available P determinations

Membranes were prepared and extracted as described by Ziadi et al. (1999). AEM strips were saturated with Cl⁻ by immersion in 1 M HCl solution and agitation in 1M NaCl solution for 1 h. Membranes were then thoroughly rinsed and kept in distilled water until soil placement. After recovery from the soil, membranes were extracted in 25 mL of 1 M KCl in test tubes by shaking for 1 h. The extract was filtered and its P concentration was determined colorimetrically by the molybdate-ascorbic acid method (Technicon AutoAnalyzer, 1976) using a Technicon segmented flow AutoAnalyzer (AAII system). Soil available P was determined by extracting 2.5 g soil in NaHCO₃ according to the method of Olsen et al. (1954). Phosphorus concentration was also determined by the colorimetric molybdate-ascorbic acid method on a Technicon segmented flow AAII system.

4.3.6 Nematode population

The soil was gently passed through a 5 mm sieve before nematode extraction using a sieving-Baermann funnel technique to extract nematodes from 60 mL sub-samples. Extracted nematodes were heat-killed and preserved in 4% formalin prior to counting in a 4 cm x 4 cm gridded counting dish. Total number of nematodes was determined with an inverted microscope at 40X. Then, transects were made through the counting dish, and the first 100 nematodes in each sample were observed for identification at 200X and 400X to the genus and classified into bacterivorous, fungivorous, omnivorous or predacious nematode trophic groups (Yeates et al., 1993; Ingham, 1994). The reciprocal

version of the Simpson's index of diversity was calculated for the nematode community as follows (Washington, 1984):

$$\text{Simpson (Si)} = 1/3P_i^2 \quad [4.1]$$

where P_i is the relative abundance of the i 'th taxon in the sample. Nematode population was identified by Dr. Tom Forge, Agriculture and Agri-Food Canada, Agassiz, British Columbia.

4.3.7 Statistical analysis

The significance of treatment effects on soil-related variables was assessed using repeated-measures ANOVA (PROC MIXED) (Wang and Goonewardene, 2004) using SAS Institute, Cary, USA. The Wilk's Lambda test was used to detect significant treatment effects. The significance of treatment effects on plant-related variables was analysed by ANOVA. The LSD test was used for treatment means comparison except for dry matter yields which were compared using 'a priori' orthogonal contrasts (Chew, 1976). Discriminant analysis of the combined PLFA profiles and nematodes class data sets was performed after transformation and standardization (Legendre and Legendre, 1998) and canonical scores were plotted to illustrate the influence of P fertilization rate on soil microbial community composition. Correlation analyses were conducted and Pearson correlations between plant biomass, root colonization and P uptake were calculated (Legendre and Legendre, 1998) using Systat v. 10 (Point Richmond, USA). The data were tested for normality using Shapiro-Wilk's test and non-normal data were transformed prior to analysis, as required by the tests. The threshold used for rejection of null hypotheses was $P < 0.05$.

4.4 Results

4.4.1 Plant yield

Total dry matter yield of the two types of plant stands responded differently to P fertilization (Fig. 4.1). Fertilization treatments did not significantly influence the yield of alfalfa monoculture; but the yield of alfalfa-RWR dual culture was reduced at low soil P fertility. Dual culture yield was 32% and 33% lower than that of alfalfa mono culture at 0P ($P = 0.04$) and at 20 P ($P = 0.02$) respectively. There was no significant P-fertilization effect on the proportion of alfalfa and RWR biomasses in dual culture stands, or on the biomass of these components of the stands, when tested separately.

4.4.2 Soil resource-related variables

Phosphorus fertilization influenced soil resource-related variables (Table 4.1). Soil phosphate flux increased with rates of P fertilization ($P = 0.0001$). Increase in soil P fertility with increased P fertilization rates was also shown by Olsen-P extraction which revealed mean soil available P values of 5.80, 35.2 and 99.6 mg kg⁻¹ soil at 0P, 20P and 40P, respectively. Fertilization had similar effect on soil P availability in mono and dual culture, as indicated by the absence of interaction effect. Gravimetric soil water content did not change with P fertilization rates (Table A.3, $P = 0.9$) or between stand's species composition (Table A.4, $P = 0.7$) treatments.

4.4.3 Soil microbial community

Phosphorus fertilization treatments modified soil microbial community structure only in alfalfa-RWR stands, as revealed by discriminant analysis of soil PLFA profiles ($P = 0.004$) (Fig. 4.2). The effect of P fertilization rates on soil microbial community

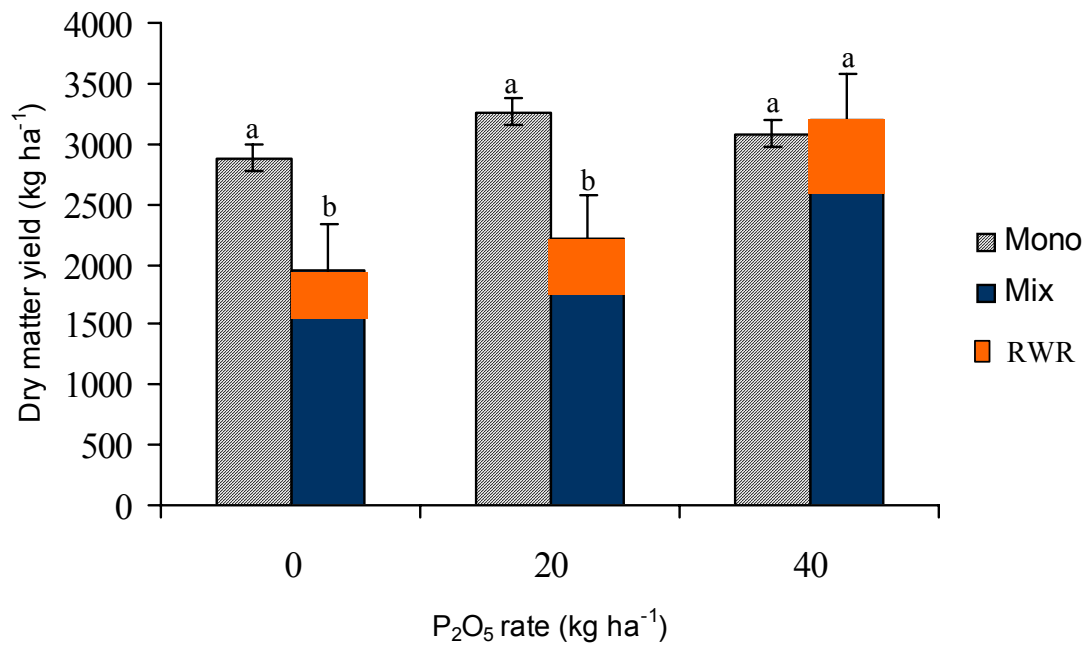


Figure 4.1 Mean dry matter yield of alfalfa and alfalfa-Russian wildrye dual culture averaged over time as influenced by P fertilization. Bars with different letters are significantly different at $P < 0.1$ and $n = 12$ (protected Fisher's LSD). Bars represent standard error.

Table 4.1 Phosphate flux, AMF root colonization and plant P concentration as influenced by P rates

P rate	PO ₄ ⁻ flux (x 10 ⁻³) (µg cm ⁻² d ⁻¹)	AMF root colonization (%)	Plant P (%)
0	0.34 ^b ± 0.2	43.2 ^a ± 4.08	0.17 ^b ± 0.006
20	0.67 ^b ± 0.1	35.1 ^b ± 2.87	0.20 ^a ± 0.005
40	2.30 ^a ± 0.4	35.6 ^b ± 3.19	0.22 ^a ± 0.007

*Means and standard errors (SE). Different letters in the same column indicate significant differences at *P* < 0.05 (protected Tukey's test), n = 24.

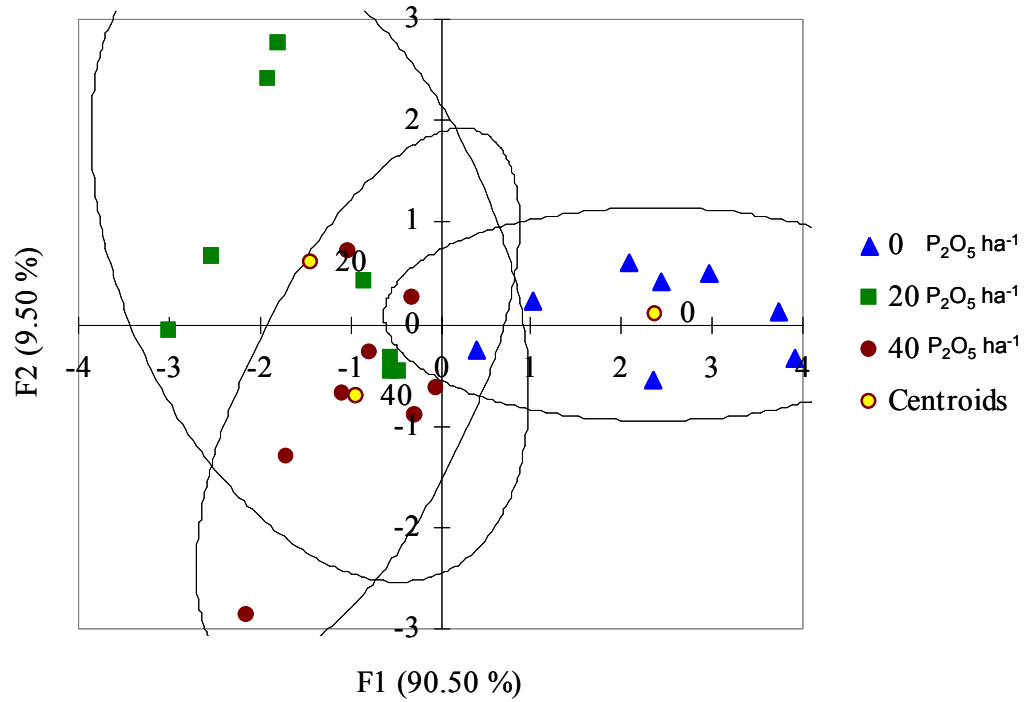


Figure 4.2 Discriminant analysis of the PLFA bacteria and fungi and nematodes (total, mycophagous, omnivorous, bacteriovorus, plant parasitic) for dual culture forage stand averaged over time. The larger circle indicate that PLFA biomarkers categorized at different P levels $P= 0.006$ and $n = 24$.

structure was detected by a model where six PLFA indicators (a-15:0, 15:0, 16:0, 16:1 ω 5, 17:0 and 2OH-16:0, and two nematode classes (omnivorous and parasitic) were significant in explaining the variability measured. The percentage of AMF root colonization decreased significantly with P rates (Table 4.1). It was also significantly lower in alfalfa-RWR dual culture (25.7%) than in alfalfa monoculture (34.1%). Root colonization by AMF was negatively correlated with total yield ($r = -0.50$) and with total P uptake ($r = -0.47$) in alfalfa-RWR stand in dual culture. The AMF colonization in monoculture was 34.1% as compared to 25.7% in dual culture.

None of the nematode groups studied was significantly affected by P fertilization rates. However, the number of fungivorous, omnivorous and total nematodes was significantly higher in stands with RWR than in alfalfa monoculture (Table 4.2). Plant parasitic nematodes were higher in alfalfa-RWR dual culture but did not vary significantly from those in alfalfa monoculture (Table 4.2). Simpson nematode diversity index, calculated at the genus level of resolution varied with crop treatments.

4.4. Discussion

Our results indicate that, under our experimental conditions, RWR selected for a soil microbial community that negatively impacted plant productivity. The RWR when grown in dual culture increased the competitive interaction between RWR and alfalfa to the benefit of alfalfa. The results provide empirical evidence of AMF involvement in negative feedback in the field, which was mediated at least in part by the proliferation of fungivorous and omnivorous nematodes in presence of RWR.

Arbuscular mycorrhizal fungi are symbiotic organisms playing an important role in plant P acquisition. Their mycelia increase the effective surface area for absorption of

Table 4.2 Number of nematodes per 100 g dry soil in mono and dual culture forage stands in the top soil layer (0-7.5 cm)

Crop	Total nematode	Fungivorous	Bacteriovorus	Omnivorous	Total plant parasitic nematodes	Shannon-Weiner index	Simpson index
Monoculture	680 b	170 b	380 a	100 b	30 a	7.19 b	5.12 b
Dual culture	902 a	199 a	275 a	379 a	48 a	9.56 a	7.14 a
±SE	68	45	ns	40	ns	0.48	0.42

Standard errors (SE). Different letters in the same column indicate significant differences at $P < 0.05$ (protected Tukey's test), $n = 12$.

root systems (Jakobsen, 1986; Liu et al., 2007). The development of AMF is controlled by soil P availability, with higher levels decreasing AMF root colonization (Bååth and Spokes, 1989; Covacevich et al., 2007). The AMF symbiosis is a buffering mechanism; it shrinks and expands when needed to allow optimal growth and P nutrition of host plants in a range of soil P fertility conditions.

The composition of plant communities is the outcome of direct competition between plants for light and soil resources as influenced by the complex interactions existing between plants and associated soil microflora (Reynolds et al., 2003). The influence of soil microbial communities on plant diversity may result from direct interactions between plant roots and pathogens (Bruehl, 1987), herbivores (Brown and Gange, 1992) and mutualists (Smith and Read, 1997). Arbuscular mycorrhizal fungi forming mutualistic associations with plants are also vulnerable to grazing by soil animals (Warnock et al., 1982; Fitter and Sanders, 1992). Grazing can disrupt AMF hyphal networks, decrease AMF root colonization of plants, and hence interfere with plant nutrition. A few studies have investigated the effect of fungivorous nematodes on the AMF symbiosis (Warnock et al., 1982; Harris and Boerner, 1990; Bakhtiar et al., 2001).

Annual applications of 20P and 40P increased soil available P levels and reduced the percentage of mycorrhizal root colonization in our experimental plots (Table 4.1), in agreement with previous reports (Covacevich et al., 2007). In monoculture alfalfa stands, increased AMF development compensated for lower levels of soil available P and, as a result, the P fertilization treatments did not influence plant biomass production (Fig. 4.1). Although similar variation in soil available P levels were induced by fertilization

treatments in alfalfa-RWR dual culture, no AMF-derived compensation mechanism was seen and plant biomass was reduced under low soil P fertility. This suggests the malfunctioning of the AMF symbiosis in the alfalfa-RWR stands.

The lower total yield of alfalfa-RWR dual culture as compared to that of alfalfa stands at low P fertility was associated with lower level of AMF root colonization and change in soil microbial community structure. In particular, alfalfa-RWR stands hosted a higher number of fungivorous and omnivorous nematodes, which also consume fungal hyphae. This indicates that the decrease in AMF root colonization observed in our dual culture is likely due, at least in part, to nematode feeding on hyphae and fragmenting the AMF hyphal network in soil, reducing its infectivity and function.

Negative interactions between organisms have generally been associated with antagonistic effects of predators and parasites (Chesson, 2000), thus it is likely that mutualistically interacting species may generate negative feedback (Bever, 1999; Bever, 2002). Warnock et al. (1982) reported that reduction in AMF root colonization with increasing collembolan density was associated with a decline in P inflow to roots from 6.8 to 1.7 $\text{pmol m}^{-1} \text{s}^{-1}$, in pot experiments. Harris and Boerner (1990) also reported decreases in AMF root colonization and plant growth with increasing grazing pressure by collembola. A few studies have investigated the effect of fungivorous nematodes such as *Aphelenchus avenae* on the AMF symbiosis. The percentage of root colonization and AMF spore number significantly decreased with an increase in nematode density (Hussey and Roncadori, 1981; Bakhtiar et al., 2001). Reduction of the nematode population in a short-grass prairie with carbofuran resulted in a 6 to 10 fold increase in AMF colonization of *Bouteloua gracilis* (Ingham et al., 1986).

Predation by nematodes can impair the function of the AMF symbiosis through reduction of AMF root colonization level and fragmentation of AMF hyphal network in soil, limiting the contribution of AMF to plant nutrition. But, the C drain created by the sustained consumption of AMF hyphae by nematodes can effectively change the nature of an AMF symbiosis from mutualistic to parasitic, as observed in our experiment. The negative relationship between AMF root colonization level and yield only seen in alfalfa-RWR dual culture suggests that the inclusion of RWR in alfalfa stands enhanced nematode proliferation and feeding pressure on AMF, turning the AMF symbiosis into a parasitic relationship. It is also possible that reduction of AMF colonization in dual culture could be due to production plant defence compounds against nematode attack (Vierheiling, 2004).

Plants experiencing P deficit invest more C in the development of AMF hyphal networks (Ryan et al., 2000; Covacevich et al., 2006). Where AMF hyphae are consumed by soil animals, disrupted AMF hyphal networks do not assist plant P uptake effectively and plants experiencing P shortage invest C in AMF symbiotic development with no return. The significantly lower content of plant P under reduced P fertilization only in dual culture is indicative of the disruption in P supply by mycorrhizae to plants.

The forage yield suppression observed in presence of RWR under low P fertility seemingly results from a C drain created by fungivorous nematode. Our results corroborate with the findings of Salawu and Estey (1979) that reported 40% decrease in shoot weight of mycorrhizal plants in presence of *A. avenae*. We observed over 30% reduction in biomass production in alfalfa-RWR stands grown at 0P and 20P. Giannakis and Sanders (1990) also reported reduced yield and lower shoot P content in AMF red

clover when inoculated with *Aphelenchoides composticola*. Finlay (1985) and Parkinson et al. (1979) reported similar findings from experiments on collembolan species, also consumers of AMF.

Previous research on the interaction between AMF and fungivorous nematodes reported in the literature (Salawu and Estey, 1979; Giannakis and Sanders, 1990) involved the direct manipulation of nematode populations as treatments. In contrast to these experiments, we show that RWR is a possible cause for increase in fungivorous nematode populations and that the inclusion of RWR in forage stands with alfalfa may result in negative feedback on plant productivity in presence of AMF.

From our results and that of others, it appears that the negative impact of increased fungivorous nematode population was larger on alfalfa-RWR dual culture than alfalfa alone. Usually alfalfa is an inferior competitor and gradually disappears when grown in mixture with grasses and in particular, with grasses and in particular, with RWR (Kilcher and Heinrichs, 1966). We found that, alfalfa persisted in our long-term alfalfa-RWR dual culture, seemingly due to more severe AMF hyphal grazing by nematodes in the root zone of RWR than that of alfalfa. A differential effect of nematodes on alfalfa and RWR could be explained by the different rooting depth of these species. Alfalfa has a much deeper root system than RWR. Arbuscular mycorrhizal fungal diversity differs with soil depth (Oehl et al., 2005) and the AMF hyphal networks associated with these plants may also vary. Nematode grazing in the surface layers of the soil would have less impact on a deep-rooted species such as alfalfa. The possibility of variable impact of fungivorous nematode grazing on AMF plants with different rooting habit is an interesting question that could be addressed in further research.

4.5 Literature Cited

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5. Preface

The work has been published in *Applied Soil Ecology* (Atul-Nayyar, C. Hamel, G. Lafond, B. Gossen, K. Hanson, J. Germida. 2009. Soil microbial quality associated with yield reduction in continuous-pea. *Appl. Soil Ecol.* 43: 115-121). This study compares the practice of monocropping and rotation cropping under different N regimes. The work offered an opportunity to look into the influence of these cropping practices on soil microbial community, AMF and soil functioning. The work was co-supervised by C. Hamel and J. Germida while G. Lafond, B. Gossen and K. Hanson (co-authors) provided their feedback for improving the manuscript. In addition, G. Lafond initiated and maintained the experimental site and B. Gossen evaluated root rot symptoms. I prepared a research proposal, plan sampling, lead the sampling team, processed and analysed most samples, coordinated analytical work done in AAFC service chemistry laboratory and prepared the manuscript.

5. SOIL MICROBIAL COMMUNITY ASSOCIATED WITH YIELD REDUCTION IN CONTINUOUS PEA

5.1 Abstract

The negative impact of continuous production on soil productivity has been demonstrated but is still not well understood in continuous-pea. The impacts of continuous pea on soil health and functioning, as compared to pea-wheat rotation, were assessed as part of an 11-year study conducted on a thin Black Chernozemic soil to understand how crop rotation affects soil microbial communities, nutrient availability, and pea nutrition and productivity. Soil and plants from the field pea phase of the rotations receiving one of three N treatments (0, 20, 40 kg N ha⁻¹) were sampled three times during the growing season of 2005. Reduction in pea plant productivity in the continuous-pea system seemed attributable to multiple causes. In continuous-pea, tissue N and P concentrations were reduced by over 10%, and tissue Fe and Mn concentrations were increased two-fold and 1.3-fold, respectively. High tissue micronutrient (Fe, Mn, and Zn) concentrations were related to the abundance of the Gram negative bacteria phospholipid fatty acid (PLFA) biomarker 2OH-14:0. In the continuous-pea rotation, the abundance of beneficial Gram positive bacteria and AMF was reduced as indicated by phospholipids fatty acid biomarkers, and the percentage of AMF root colonization was lower. These differences in soil microbial biodiversity could be related to increased susceptibility of continuous-pea to root rot injury, as continuous-pea root density was reduced 2-fold as compared to pea-wheat rotation. Fusarium root rot was more severe in the continuous-pea than pea-wheat

rotation (assessed in 2001 and 2008). Reduced soil organic carbon levels, and dehydrogenase, phosphatase and urease activity in continuous-pea, revealed a negative impact of continuous-pea on nutrient cycling. Greater residual NO₃ level in the soil profile indicated inefficient use of N and increased risk of N losses with continuous-pea.

5.2 Introduction

Fallow-cereal cropping rotations, which were widely used across western Canada for many years, have a negative impact on soil through erosion, low carbon inputs and depletion of fertility (Janzen, 2001). However, cropping intensity and diversity in the Canadian prairies have increased with adoption of conservation tillage systems that improve water conservation and crop yield (Lafond et al., 2006). Diversified crop rotations involving oilseed and pulse crops make better use of available water and prevent build-up of diseases and other pests (Bailey et al., 2001).

Field pea (*Pisum sativum* L.) is grown in rotation with cereal and oilseed crops on the Canadian prairies. Pea acreage has increased to 1.5 million ha in 2008 from 0.14 million ha in 1990 in Canada. In the drier regions of the prairies, growing field pea in rotation with wheat (*Triticum aestivum* L.) offers a number of important advantages. Pea grains are a major source of dietary protein for human and livestock consumption, and export markets are strong. Also, a field pea crop fixes atmospheric N, which reduces dependence on N fertilizers and has a beneficial effect on soil fertility (Soon and Clayton, 2002; Soon and Arshad, 2005).

Wheat yields are often higher after a field pea crop than when following cereal or oilseed crops (Soon and Clayton, 2002). Research on cropping rotations has focussed on determining: i) the fertilization replacement values of field pea in a crop rotation (Wright,

1990; Beckie and Brandt, 1997; Soon and Arshad, 2005); ii) the impact of field pea on soil physical properties such as aggregate size distribution and bulk density (Grant and Lafond, 1993; Robinson et al., 1994); iii) its impact on nutrient and water use efficiency (Gan et al., 2003; Lafond et al., 2006); and iv) the suppression of disease and insect pests (Bailey et al., 1992; Bailey et al., 2001). The rotational benefits of field pea crop on subsequent wheat crop have been attributed to soil nutrient and water supply, soil physical properties, interruption of pest cycles and stimulation of beneficial microorganisms (Grant and Lafond, 1993; Bailey et al., 2001; Gan et al., 2003).

Field pea and wheat in Saskatchewan have several fungal root rot pathogens in common, e.g., *Fusarium avenaceum* (Fernandez et al., 2008). However, *Pythium* spp. are important early season root pathogens of pea (Hwang et al., 2001) and other pulse crops, but are not important pathogens on wheat in this region. The underlying mechanisms for the beneficial impact of crop rotation on plant diseases (Kirkegaard et al., 2008) and improved yield when growing pea and wheat in a rotation are not yet well understood.

The objective of this study was to document the effect of pea in rotation and monoculture on the soil microbial community and functioning. Rotation effects were examined in an 9-year old field experiment set up in Saskatchewan, Canada.

5.3 Materials and Methods

5.3.1 Site description

The experiment was conducted on a Black Chernozemic (Udic Boroll) soil located at the Agriculture and Agri-Food Canada Research Farm in Indian Head, Saskatchewan, Canada. The soil is a heavy clay with 55.5% clay, 20.4% silt and 24.1% sand with a near neutral pH of 7.5 (soil: water, 1:1 w/w). Soil EC was 0.50 mS cm⁻¹ and

organic matter 4% when the experiment was initiated in 1995. It was set up as a randomized complete block design with four replicates of N fertilization rates and crop rotation. Soil sampling was done in 2005 in the pea phase of each rotation. Crop rotation consisted of field pea cv. Eclipse grown either continuously (continuous-pea) or in rotation (pea-wheat) with wheat cv. Prodigy. Field pea was sown at the rate of 194 kg ha⁻¹ on 26 April and was harvested on 7 September, 2005. All fertilizers were side-banded together using a ConservaPak no-till plot seeder. All the plots received 11 kg ha⁻¹ of P₂O₅ as mono-ammonium phosphate with an analysis of 11-52-0 (providing 5.5 kg N ha⁻¹). The N applied through was 5.5 kg ha⁻¹. Three rates of N fertilizers (0, 20, and 40 kg N ha⁻¹) were applied to both continuous-pea and pea-wheat rotation. The herbicide triallate, granular formulation, was surface applied the previous fall at a rate of 1.7 kg ai ha⁻¹. The in crop herbicide imazamox (35%) and imazethapyr (35%) was applied at 17 g ha⁻¹ in pea. Total precipitation and average temperature was recorded for the year 2005 (Table A.5)

5.3.2 Sampling and plant analyses

The experiment was sampled four times during the 2005 growing season. The soil was sampled before seeding to assess NO₃-N in the soil profile (0 to 15, 15 to 30, 30 to 60 and 60 to 120 cm). On 29 June, 18 July and 11 August, soil samples from the top 0 to 7.5 cm soil layer were taken with a bulb planter. Five cores per plot were collected and bulked together. The composite sample was then run through a 2-mm sieve. After sieving, the soil was divided into two parts: one part was stored at -20°C for fatty acid analysis and the other was stored at 4°C for a few days until enzyme assays and soil organic C assessments were completed (Baccanti and Colombo, 1992). Arbuscular

mycorrhizal root colonization and root abundance on pea were assessed on soil cores (5-cm diam., top 7.5 cm soil layer) taken at two randomly selected locations per plot using a manually operated soil sampler. Roots were extracted from these cores, washed, and parsed into two subsamples of known weight, one for mycorrhizal root colonization assessment and the other for determination of root abundance. Root abundance was expressed in mg dm^{-3} of soil. The percentage of AMF colonization in the roots was determined by the gridline intersects method under a dissecting microscope (Giovannetti and Mosse, 1980). Roots were boiled in a 10% KOH solution for 10 min, rinsed with tap water, and stained by boiling for 3 min in ink-vinegar solution and then cut into 1-cm lengths (Vierheilig et al., 1998) prior to microscopic examination.

Plant shoots were taken from two randomly selected 1-m row lengths in each plot, at all three sampling dates. They were dried at 40°C to a constant weight, and dry matter yield was recorded. The dry shoots were ground, digested with $\text{H}_2\text{SO}_4/\text{Se}/\text{Na}_2\text{SO}_4$ (Varley, 1966) and analyzed for tissue N (Noel and Hambleton, 1976) and P (Milbury et al., 1970) on a segmented flow auto-analyzer (Technicon, AAII System, Tarrytown, NY), and K concentrations, using atomic absorption spectrophotometry (Hitachi, Japan). Uptake of N, P and K by pea was calculated by multiplying pea biomass (kg ha^{-1}) by the concentration of each individual nutrient, and expressed as kg ha^{-1} . Ground plant samples were also digested with $\text{HClO}_4/\text{HNO}_3$ (Jones, 1991) and analysed for Ca, Mg, Fe, Mn, and Zn using atomic absorption spectrophotometry (Hitachi, Japan).

Three sets of three anion exchange membranes (AEM, #200253, Ionics, Watertown, MA) were inserted at 7.5 cm depth in the soil of each and all plots through a slit opened with a small shovel. These sets were incubated during one of three periods: i)

29 April – 29 June, ii) 29 June – 18 July, and iii) 18 July – 11 August. After incubation, each set of membrane was replaced with a new set. The membranes removed from the soil were rinsed with distilled water to wash off adhering soil, and analyzed for soil PO₄ and NO₃ flux determinations as described below.

5.3.3 Soil nutrient availability

Membranes were prepared and extracted as described by Ziadi et al. (1999). AEM strips were saturated with Cl⁻ by immersion in 1 M HCl solution and agitation in 1 M NaCl solution for 1 h. Membranes were then thoroughly rinsed and kept in distilled water until soil placement. After recovery from the soil, membranes were extracted in 25 mL of 1 M KCl in test tubes by shaking for 1 h. The extract was filtered and its P concentration was determined colorimetrically (Milbury et al., 1970), and NO₃-N concentration was measured by hydrazine reduction (Anonymous, 1987) using a segmented flow auto-analyzer (AAII System, Tarrytown, NY).

Soil available P was determined by extracting 2.5 g soil with NaHCO₃ according to Olsen et al. (1954). P concentration was also determined by colorimetry with the segmented flow auto-analyzer (Milbury et al., 1970).

5.3.4 Soil microbial community

Fatty acids in the phospholipid (PLFA) fractions of soil extracts were analyzed and some of them used as indicators of active soil microbial biomass, as per the methods described by Hamel et al. (2006). Total soil lipids were extracted from 4 g of soil (dry weight equivalent) in dichloromethane (DCM): methanol (MeOH): citrate buffer (1:2:0.8 v/v). Fatty acid methyl esters were analyzed using a gas chromatograph (GC) equipped with a CP-8400 autosampler and a flame ionization detector (FID) (3900, Varian,

Neitherlands) using Helium as the carrier gas (30 ml min⁻¹) and a 50-m Varian Capillary Select FAME # cp7420 column. Sample (2 µl) injection was in a 5:1 split mode. The injector was held at 250°C and the FID at 300°C. The initial oven temperature of 140°C was held for 5 min, raised to 210°C at a rate of 2°C min⁻¹, then raised from 210°C to 250°C at a rate of 5°C min⁻¹ and held for 12 min.

Identification of peaks was based on the comparison of retention times to known standards (Supelco Bacterial Acid Methyl Esters #47080-U, plus MJS Biolynx #MT1208 for 16:1ω5). The abundance of individual PLFAs was expressed as µg PLFA g⁻¹ dry soil. Amounts were derived from the relative area under specific peaks, as compared to the internal standard (19:0) peak value, which was calibrated according to a standard curve made from a range of concentrations of the 19:0 FAME standard dissolved in hexane. Fatty acids were named according to the ω-designation described as follows: total number of carbons followed by a colon; the number of double bonds; the symbol ω; the position of the first double bond from the methyl end of the molecule. Cis and trans isomers are indicated with c or t, respectively. Methyl (meth) and hydroxy (OH) groups are labeled at the beginning, where applicable. Iso and anteiso forms are indicated by i- and a-, respectively.

Individual fatty acids have been used as signatures for various groups of microorganisms (Pankhurst et al., 2002; Hamel et al., 2006). The FAME 18:2, 18:1c and 18:1t were used as indicators of saprophytic fungal biomass (Petersen and Klug, 1994; Frostegård and Bååth, 1996), and FAME 16:1ω5 as an indicator of AMF (Spring et al., 2000; Balser et al., 2005). The FAMES 17:0, ante 15:0, 15:0, i-17:0 + 17:0, 15:0, i-15:0, i-16:0 were used as indicators of Gram positive bacteria and FAMES 3OH-12:0, 2OH-

14:0, 2OH-16:0, 3OH-14:0 as indicators of Gram negative bacteria (Zelles, 1997; Olsson, 1999; Otto et al., 2005).

5.3.5 Soil enzyme assays

Soil enzyme activities were determined for three groups of enzymes: dehydrogenase, phosphatase and urease. Dehydrogenase was assayed as reported by Casida et al. (1964). One mL of 3% triphenyltetrazolium chloride (TTC) and 2.5 mL of dd H₂O was added to test tube containing 6 g of soil at field capacity and 60 mg of CaCO₃. After 24 hours of incubation at 37°C, 10 mL of methanol was added and the content of each tube was mixed thoroughly and filtered through a Whatman No. 42 filter paper by washing with 50 ml of methanol. The optical density (O.D.) of the filtrate was measured by spectrophotometry at 485 nm.

The phosphatase assay followed the method of Eivazi and Tabatabai (1977). Four ml of modified universal buffer, 1 mL of p-nitrophenyl phosphate solution and 0.2 mL of toluene were added to 1 g of soil in test tubes (16 x 125 mm). After 1 hour of incubation at 37°C, 1 mL of 0.5 M CaCl₂ and 4 ml of 0.05 M NaOH were added; the contents were mixed thoroughly and filtered through Whatman No. 42. The yellow colored p-nitrophenol of the filtrate was measured at 400 nm.

For the urease assay, 2.5 g of soil was mixed with 1.3 mL of 0.08N urea solution into a flask, capped, and incubated at 37°C for 2 hours. Then, 25 mL of 1N KCl / 0.1N HCl solution were added before shaking for 30 min. The filtrate (Whatman No. 40 filter paper) was analyzed for NH₄⁺-N by the Kjeldahl- N method (Jones, 1984).

5.3.6 Root rot severity

The field pea phase of each treatment was rated for root rot severity in 2001 and 2008. At harvest, the roots of 10 plants per plot were dug by hand, washed, and assessed for root rot severity on a 0 to 4 scale, where 0 = healthy; 1 = small brown lesion on < 25% of the exterior circumference of the tap root; 2 = lesion on 25–49% of tap root; 3 = lesion on 50–74% of tap root; 4 = tap root girdled (75–100%) and plants wilted and dying. The fungal pathogens associated with the lesions were identified by isolation of random lesions onto PDA agar amended with streptomycin. The identification was based on production of a moderate amount of pale aerial mycelium, carmine red pigment in the media, and falcate (moon-shaped) clusters of conidia on single aeriia conidiophores.

5.3.7 Statistical analysis

Treatment effects of soil-related variables were assessed using repeated-measures analysis of variance (PROC MIXED) (Wang and Goonewardene, 2004) and the plant-related variables by analysis of variance using JMP 3.2.6 software (SAS Institute, Cary, NC). The data was tested for normality using Shapiro-Wilk's test and non-normal data were transformed prior to analysis. The plant tissue Fe and Mn concentration data could not be normalized and was analyzed using the Kruskal-Wallis test in SYSTAT 12. The least square differences (LSD) was used for treatment mean comparisons at $P \leq 0.05$. The relationship between plant variables and the soil biological environment of the two cropping systems on 18 July, when effects on plant biomass were developing, was analyzed by canonical correspondence analysis (CCA) using Euclidean distance in PC-ORD, version 4.34 (MjM Software, Gleneden Beach, OR) after standardization of the data (Legendre and Legendre, 1998). Regression analysis was conducted with JMP 7.0.1

(SAS Institute, Cary, NC) to assess the relationship between reduction in seed yield under continuous-pea as compared to pea-wheat, measured from 1995 to 2005, and the number of years since the onset of the experiment. The yield data prior to 2005 were taken from Lafond et al. (2007). The treatment effect on the categorical variable “disease incidence” was submitted to analysis of variance (Crawley, 2007) and analyzed by REML (PROC MIXED) using SAS (SAS Institute, Inc. 1996).

5.4 Results

5.4.1 Nitrogen fertilization

Nitrogen fertilization had no impact on plant and soil measured variables except for mycorrhizal root colonization, which decreased by 17% ($P = 0.001$) at 40 kg N ha⁻¹ (29.2%) as compared to the control (35.1%). In contrast, cropping system had an impact on most variables measured (Table 5.1)

5.4.2 Soil biological properties

The percentage of AMF root colonization and the abundance of PLFA 16:1 ω 5, the indicator of AMF biomass, were higher in the pea-wheat rotation than in continuous-pea (Table 5.1, Fig. 5.1). The abundance of PLFA biomarkers of saprophytic fungi, Gram positive and Gram negative bacteria were higher in the pea-wheat rotation for 9 of the 13 indicators used, and none were lower (Fig. 5.1).

Crop rotation also influenced soil microbial activity (Table 5.1). Activity assessed using the dehydrogenase assay was higher in the pea-wheat than in the continuous-pea rotation. The activity of phosphatase, a soil enzyme induced by P demand, was also higher under the pea-wheat rotation. Urease, a soil enzyme responsible for urea hydrolysis, also showed higher activity in the pea-wheat rotation.

Table 5.1 Soil nitrate (AEM NO₃) and phosphate (AEM PO₄) flux, total phospholipids (PLFA) biomarker, enzyme activity (0-7.5 cm depth) and AMF root colonization in pea growing soil under continuous-pea and pea-wheat (*n* = 36).

Parameters/Crop	Continuous- pea	Pea-wheat	S.E.*	<i>P</i> value
AEM NO ₃ (µg cm ⁻² day ⁻¹)	6.1 x 10 ⁻³	3.6 x 10 ⁻³	0.84 x 10 ⁻³	0.0001
AEM PO ₄ (µg cm ⁻² day ⁻¹)	7.8 x 10 ⁻⁵	9.5 x 10 ⁻⁵	0.01 x 10 ⁻³	ns
Total PLFA biomarker (µg g ⁻¹)	37.2	46.6	1.68	0.002
Dehydrogenase activity (µg TPF \day ⁻¹)	78.6	97.0	8.75	0.002
Phosphatase activity (µg p-nitrophenol produced g ⁻¹ hr ⁻¹)	24.6	31.1	1.45	0.0001
Urease activity (µg N g ⁻¹ soil)	4.32	5.46	0.43	0.001
AMF root colonization (%)	30.9	34.2	1.26	0.002
Soil organic C (mg g ⁻¹)	2.49	2.74	0.06	0.01

* S. E. : standard error of the means

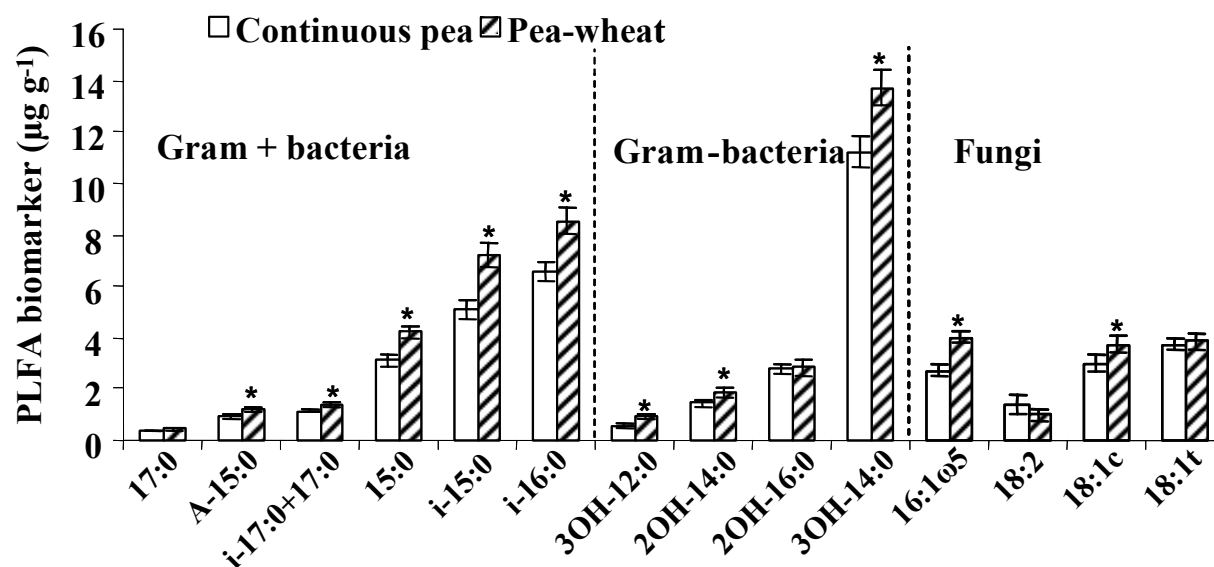


Figure 5.1 Effect of crop rotation treatments on bacterial and fungal PLFA biomarkers. The symbol * indicates that amounts of biomarker under continuous-pea and pea-wheat are significantly different at $P = 0.05$, according to ANOVA, $n = 36$; bars are standard errors of the means (protected Fisher's LSD).

5.4.3 Soil nutrient availability

The mean nitrate flux, measured by AEM during crop growth, was higher in continuous-pea than in the pea-wheat rotation, but phosphate flux was not affected by crop rotation (Table 5.1). The level of NO₃ before seeding was lower in the pea-wheat rotation than in the continuous-pea rotation, and the difference between treatments was more pronounced at the lower depths (Table 5.2). Soil extractable P content was not influenced by cropping system. Continuous-pea had 0.091 mg/mL of soil P while pea-wheat had 0.093 mg/mL of soil P.

5.4.4 Pea biomass

The field pea biomass produced under continuous-pea and the pea-wheat rotations were similar on 29 June, but 1.7 times larger in the pea-wheat rotation 20 d later, and 2.1 times larger on 11 August (Fig. 5.2). Pea-plant tissue N and P concentrations were 11 % and 15 % higher in pea-wheat than continuous-pea rotations, while concentrations of Ca, Mg, Fe and Mn were higher in continuous-pea (Table 5.3). There was no effect of crop rotation on plant Zn concentrations (Table 5.3). Plant uptake of N, P and K was also higher in the pea-wheat than in the continuous-pea rotation, a reflection of the higher biomass production with the pea-wheat rotation (Table 5.3). Increase in pea tissue Fe concentration was particularly large as it was about twice that measured in pea-wheat rotation (Table 5.3), but differences in tissues Zn concentration were not significant. The pea-wheat rotation had a grain yield 3.3 times larger, and a root system 2.0 times denser than the continuous-pea rotation (Table 5.3).

Table 5.2 Pre-seeding NO₃-N content at different soil depths under continuous-pea and pea-wheat systems. The symbols *, **, and *** indicate that differences between continuous-pea and pea-wheat are significant at $\alpha = 0.05$, $\alpha = 0.01$, and $\alpha = 0.0001$, respectively, according to ANOVA, $n = 12$.

Depth (cm)	Continuous-pea	Pea-wheat	S.E.*
0-15	115.4**	16.9	0.94
15-30	70.52*	23.4	1.03
30-60	119.52***	6.07	0.68
60-120	192.1***	3.4	0.69

* S. E. : standard error of the means

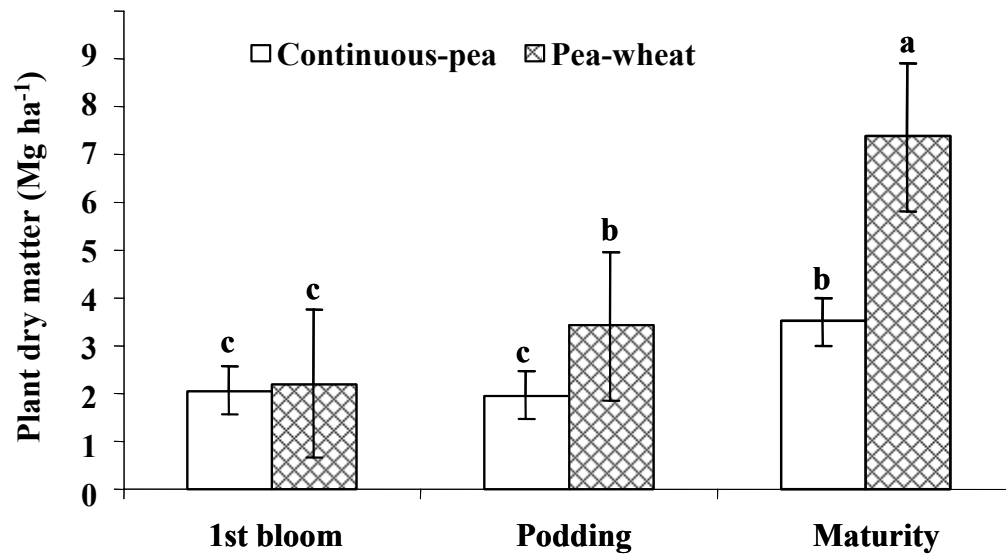


Figure 5.2 Effect of cropping system and sampling time on dry matter yield of pea. Means with different letters are different according to protected LSD test at $P = 0.05$, $n = 12$. Bars represent standard errors of means.

Table 5.3 Grain yield, root abundance, plant tissue macro and micronutrient concentrations and uptake, and grain yield in continuous-pea and pea-wheat ($n = 36$ except for root abundance, measured only in July and August 2005 $n = 24$, and yield $n = 12$).

Parameters/Crop	Continuous-pea	Pea-wheat	<i>P</i> value
Grain yield (kg ha ⁻¹)	451	1509	<0.0001
Root abundance (mg m ⁻³)	180	370	0.01
Shoot N (mg g ⁻¹)	28.3	31.7	0.002
Shoot P (mg g ⁻¹)	3.4	4.0	<0.0001
N uptake (kg ha ⁻¹)	70	121	<0.0001
P uptake (kg ha ⁻¹)	8.4	14.9	<0.0001
K uptake (kg ha ⁻¹)	44.0	83.3	<0.0001
Ca (μg g ⁻¹)	13924	12627	0.004
Mg (μg g ⁻¹)	3931	3440	0.001
Fe (μg g ⁻¹)	1101	500	<0.0001
Mn (μg g ⁻¹)	67.7	53.1	0.000
Zn (μg g ⁻¹)	14.8	15.8	ns

5.4.5 Root rot severity

Pea roots were assessed for root rot injury in 2001 and 2008. Root rot severity was lower ($P < 0.0001$) under the pea-wheat rotation and rated 1.9 in 2001 and 0.5 in 2008 on a 0-4 scale, as compared to 2.5 and 0.8 in continuous-pea in the same years. The lower incidence of root rot in continuous-pea in 2008 as compared to 2001 is not clear. *Fusarium* spp. were the predominant pathogens isolated, followed by *Rhizoctonia* spp. and a few isolates of *Pythium* spp.

5.4.6 Relationship between field pea biomass and soil microbial indicators

Canonical correspondence analysis (CCA) revealed a relationship ($P = 0.02$) between soil microbial parameters (PLFA biomarkers, mycorrhizal root colonization, and enzyme activity) and plant physiological status (biomass production, root abundance, and tissue nutrient concentrations) on 18 July. Axis 1, which explained 74% of the variability contained in the data, separated the two rotations (Fig. 5.4a). Enzyme activities and microbial biomass, as indicated by PLFA biomarkers, were positively related to plant biomass production. The only exception was Gram negative bacteria biomarkers 2OH-14:0, which were positively related to the micronutrient content of plants, and inversely related to root abundance. The reduction in seed yield under continuous-pea rotation was positively related ($P = 0.01$) to the number of years since the onset of the experiment (Fig. 5.5).

5.5 Discussion

Crop productivity along with soil organic carbon levels and soil microbial community structure and function were negatively affected by continuous cropping of

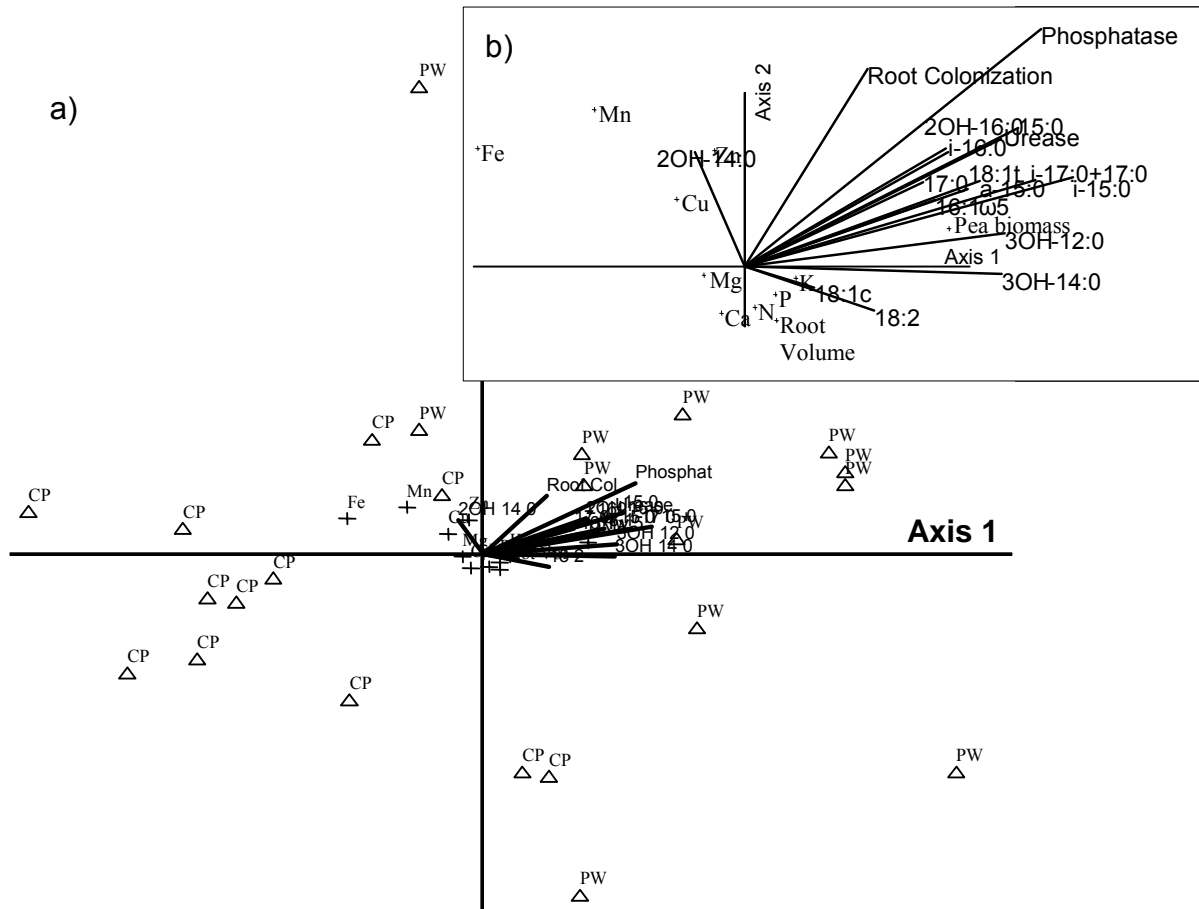


Figure 5.3a Canonical correspondence analysis (CCA) ordination plot ($P = 0.02$, $N = 24$) of the relationships between plant variables (dots) and the soil biological environment (vectors) of the two cropping systems sampled on 18 July. Plots are represented by triangles. CP= continuous-pea and PW= pea-wheat plots. Axes 1 and 2 account for 74.1% and 11.8% of the variation in the data, respectively. Figure 5.3b shows magnified representation of the relationships shown in Figure 5.3a.

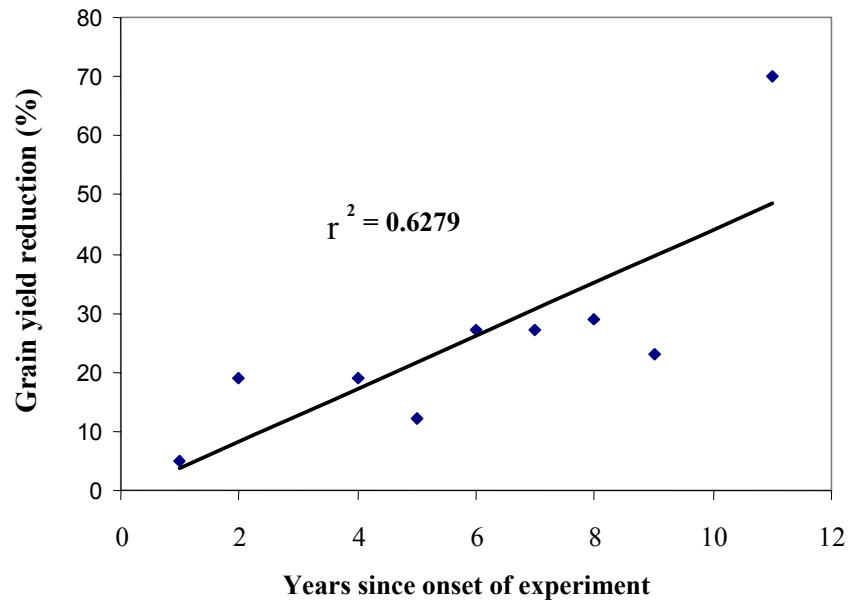


Figure 5.4 Relationship between the reduction in pea grain yield under continuous-pea as compared to the pea-wheat rotation with years since the initiation of the experiment. Yields for 1997 and 2004 are not available. Data for yields from 1995 to 2003 are from Lafond et al. 2007.

field pea as shown in the CCA ordination (Fig. 5.3). A reduction in root abundance, reductions in the concentration of N and P in plant tissues indicated that growth depression in continuous-pea involved impaired root function. The negative impact of continuous cropping was growing with the number of years of continuous-pea, suggesting that profound changes in the soil environment related to pea productivity were also occurring. Hamel et al. (2005) reported that a time lag corresponding to the restructuring of the soil microbial community was necessary for the expression of *Fusarium* crown and root rot in asparagus after a few years of cropping.

Fusarium avenaceum is likely the cause of root rot in field pea at this site. It is the dominant root rot pathogen in pea production areas in the adjacent province of Alberta (Chang et al., 2004; Feng et al., 2009), was identified from all discoloured pea roots from eastern Saskatchewan (Fernandez, 2007), and is an important pathogen of lentil and other grain legumes in the region (Hwang et al., 2000). Observations made in 2001 and 2008 showed that there was substantially (30–50%) more root rot under continuous-pea than rotational pea. The impact of early-season infection by *Pythium* spp. (Hwang et al., 2001), which preferentially attack root nodules that are important for N fixation and plant growth, could also have had an important effect on subsequent root development but would not have been captured in these late-season assessments of root discoloration. Though *Fusarium* root rot could be one possible cause for yield reduction, the low level of root discoloration seen at harvest in 2008 suggests that reduction in plant productivity under continuous pea had other causes beside *Fusarium* root rot.

Since the principle pathogenic species observed on field pea, *F. avenaceum*, is also a pathogen on wheat roots (Fernandez, 2007), differences in inoculum pressure

between the rotation systems for this pathogen are likely small. Therefore, the difference in root rot severity between the cropping systems may be due to the presence of other pathogens (e.g., *Pythium* spp.) or to differences among members of the beneficial microbial community under the two rotations. Estimates of microbial biomass based on total PLFA biomarker was generally larger under pea-wheat rotation, likely as a result of higher plant productivity, which also contributed to higher soil organic matter levels in the pea-wheat rotation (Table 5.1). One exception was the abundance of the Gram negative bacteria indicator PLFA 2OH-14:0, which was negatively associated with root abundance (Fig. 5.4). It was also positively related to plant content of Ca, Mg, Mn, and in particular Fe, which was almost two times higher in the continuous-pea than the pea-wheat rotation. Because the proliferation of pathogenic soil bacteria is usually Fe limited, the abundance of available Fe can be an important factor in the development of root diseases (Expert et al., 1996; Rahman and Punja, 2006) and might be responsible for reduced root abundance and plant productivity in continuous-pea. It is unclear if the bacterial group represented by this PLFA biomarker is involved in disease incidence or simply favoured by the conditions prevailing in continuous-pea rotation.

Differences in the abundance of beneficial microorganisms could also explain, at least in part, why root rot was less severe in pea-wheat rotation. The amount and kind of root exudates differ between plant species, and these differences can stimulate species-specific shifts in the soil microbial community (Lynch, 1990; Lupwayi et al., 1998; Welbaum et al., 2004). *Iso* and *anteiso* fatty acids are indicative of beneficial Gram positive bacteria such as *Clostridium* and *Bacillus* (Brennan, 1988; O' Leary and Wilkinson, 1988; Kaneda, 1991). Beneficial Gram positive bacteria were more abundant

in the pea-wheat rotation than continuous-pea, as indicated by the abundance of their corresponding PLFA biomarkers. Arbuscular mycorrhizal fungi were also more abundant in the pea-wheat rotation, as shown by more extensive root colonization in this rotation. The biocontrol activity of AMF is well known (St-Arnaud et al., 1995; Lioussanne et al., 2007). It appears that the pea-wheat rotation may support more beneficial microorganisms than continuous pea. The positive effect of crop rotation on the abundance of beneficial microorganisms was recently reported (Larkin and Honeycutt, 2005).

Higher root rot in continuous pea may be the consequence of Fe and Mn toxicity in rhizosphere soil. The levels of Fe in field pea tissues when grown continuously were in the toxic range (Brady and Weil, 2002; Sahrawat, 2004) and toxic levels of Fe may have directly inhibit root growth and function or acted as stresses that trigger disease. *Fusarium avenaceum* is known as an opportunistic pathogen causing damage in plants weakened by stress (Hwang et al., 2000). The cause of elevated levels of Fe in the continuous-pea rotation is unclear, but is likely related to the activity of soil microorganisms which could be pathogenic. Soil microorganisms can trigger plant defence reactions and the production of phenolics, which mobilizes soil Fe. Plant phenolics, which are produced in response to pathogenic attack, are ligands complexing metallic cations that may increase their bioavailability to plants (Robin and Stangoulis, 2003). Several soil microorganisms can also directly increase the availability of the metallic nutrient through the production of chelating molecules (Expert et al., 1996; Robin et al., 2008).

Another explanation for reduced plant productivity, under continuous pea is that more virulent strains of root pathogens may have accumulated in the continuous-pea rotation. Rotation did not influence the genotype or virulence of *Rhizoctonia solani* in a previous study on rice (Banniza et al., 1999), but this possibility may merit further study.

Crop rotation influences soil microbial communities (Grayston et al., 1998; Wardle, 1998; Spehn et al., 2000; Steer and Harris, 2000; Larkin, 2008) and soil quality in terms of nutrient availability and plant productivity (Bünemann et al., 2008; Kirkegaard et al., 2008). Plants have a major influence on soil heterotrophic microorganisms as they are their main source of C and energy. Here, 11 years of continuous pea crops repressed soil microbial biomass and degraded soil functioning. A reduction of about 20% in the activity of all enzymes, dehydrogenase, phosphatase and urease, indicated a reduced rate of nutrient cycling in soil under continuous-pea. Growth limitation in continuous-pea prevented plant uptake of N increasing risks of nitrate loss. Nitrogen fertilization of pea, an N-fixing crop, is not a recommended practice, and leaching loss in a well managed field would be lower than in this study. However, our results illustrate that the reduced capacity of plants to take up nutrients, when grown in unhealthy soils, may lead to nutrient loss. Such losses have potential impacts on environmental soil quality.

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6. Preface

At a long-term experimental site located in Agassiz BC, a region with abundant precipitation and mild climate, the practices of organic and mineral fertilization were compared. It offered an opportunity to define the influence of fertilization practices on AMF and soil microorganisms under a climate contrasting with that of Praries where other studies have been conducted. The study demonstrated that application of manure modifies soil microbial community structure and that manure containing P in excess of crop need can potentially harm the environment. The experiment was initiated and maintained by S. Bittman. The work was co-supervised by C. Hamel, J. Germida. T. Forge evaluated nematode population on experimental site. I prepared a research proposal, plan sampling, lead the sampling team, processed and analysed most samples, coordinated analytical work done in AAFC service chemistry laboratory, interpreted the data and prepared a manuscript for publication in Soil Biology and biochemistry.

6. LONG-TERM APPLICATION OF DAIRY MANURE SLURRY COMPARED TO COMMERCIAL FERTILIZATION DOES NOT IMPROVE THE PRODUCTIVITY OF FORAGE CROP IN MONROE SOIL

6.1 Abstract

Dairy manure slurry (DMS) is applied to crops as an alternative to mineral fertilizer and to improve soil organic matter richness. However, continuous and high levels of DMS can have environmental impacts. This study describes the impact of DMS and ammonium nitrate (AN) on soil quality parameters and potential environmental risks. Soil samples (0 to 7.5 cm depth) were collected three times from plots supporting tall fescue (*Festuca arundinacea* Scherb. var. *Festorina*) after DMS or AN each was applied in the amount required to contribute 50 or 100 kg mineral N ha⁻¹ (DMS₅₀ or DMS₁₀₀, and AN₅₀ or AN₁₀₀) up to four times per year for 9 consecutive years, followed by one year of stand renovation through re-seeding without fertilizer application just prior to the year of study. Tall fescue yields were similar between DMS and AN at the same level of NH₄-N (50 and 100 kg N ha⁻¹). Greater soil SOC, SON, light fraction of organic matter, microbial biomass and enzymes were observed in the DMS than in AN and unfertilized control treatment. There was a definite shift in the microbial community composition as characterized by phospholipids fatty acid (PLFA) profiles. The DMS₁₀₀ treatment resulted in an increase in phosphate flux as compared to AN and unfertilized control, particularly at last harvest (12 November, 2004), indicating increased environmental risk. DMS₁₀₀ and AN₁₀₀ applied before the last harvest also increased nitrate flux (AEM-NO₃) as compared to DMS₅₀ and AN₅₀. We conclude that long-term use of DMS in multi-cut tall

fescue increases soil quality indicators and that increase in yield in DMS plots seems to be of biological origin. However, DMS applied during September to the last harvest enhances potential environmental risk.

6.2 Introduction

Animal manure has been traditionally used to improve soil fertility and physical properties (Haynes and Naidu, 1998; Khan et al., 2007; Franke et al., 2008;), crop nutrition, and yields (Matsi et al., 2003; Zhang et al., 2006; Lithourgidis et al., 2007; Nyiraneza et al., 2009). The value of manure as a source of N can be very high (Lithourgidis et al., 2007) and manure is an attractive alternative to mineral fertilizers especially on animal production farms. Important quantities of manure can be generated from these farms and N is needed for good hay production. Perennial forage crops utilize manure better than annual crops as grasses generally have large capacity to absorb N and can receive several applications over the growing season before each growth cycle. Also, there is less risk of nutrient loss through run-off because of the year-round ground cover of forage crops.

Zhang et al. (2006) reported that liquid cattle manure was better than solid cattle manure in promoting brome grass and oat yield. This is probably due to greater content of immediately available N in liquid than solid manure. Approximately half of the N in dairy manure slurry (DMS) is in ammonium form, the remainder being in organic forms (Bittman et al., 2007). The availability of DMS-N to crops is usually lower when surface applied as compared to injected in soil. This is due to losses through volatilization of NH_3 from manure when it is surface applied and to the mineralization of organic N, which is not always synchronized with plant demand, leading to further losses of N.

Injection of liquid cattle manure below the soil surface reduces the risk of NH_3 volatilization loss as compared to surface application and may result in the production of yields similar to those produced using mineral N sources at the same rate (Matsi et al., 2003). Using surface banding of DMS, Bittman et al. (2007) showed that band application of DMS could improve the agronomic effectiveness of the materials. Crop recovery of applied manure reached 77% of total N applied in DMS. The higher yield of DMS-fertilized tall fescue stands, as compared with a mineral fertilization system receiving the same amount of mineral N annually, can be at least partly explained by residual N released from the mineralization of DMS organic fractions from previous years' applications. Manures have a residual nutritional effect, releasing nutrients in the year following application, as the decomposition of organic material often takes longer than a cropping season (Cusick et al., 2006; Zhang et al., 2006). The authors also observed that approximately one third of the applied DMS-N was being stored in soil organic matter (Bittman et al., 2007) suggesting that aggradation of soil organic matter might also enhance soil productivity under DMS fertilization.

The beneficial effect of manure application is not limited to the provision of N to crops. Repeated applications of manures may result in significant increase in soil P fertility and availability of other nutrients (Schröder et al., 2005). Literature on nutrient input through organic and conventional amendments from short and long-term studies reports the higher P, K, Ca and Mg fertility in soils receiving manure than inorganic fertilizers (Clark et al., 1998). Organic amendments usually increase the amount of soil C which may increase microbial biomass (Dhillon, 1997; Peacock et al., 2001; Ebhin Masto et al., 2006) and the activity of various enzymes (Allison et al., 2007; Li et al.,

2008). Most of the processes occurring in soils are mediated by microorganisms. The application of DMS can modify soil microbial community size and structure (Stark et al., 2008) thus can affect soil functioning. Soil biological properties can serve as early and sensitive indicators of the response of the agroecosystems' to soil management practices (Schloter et al., 2003; Bending et al., 2004).

Large amounts of DMS are produced in south coastal region of British Columbia. Perennial forage crops grown on the undulating deposits of the Fraser River Valley make good use of DMS in responding well, even to annual rates of application as high as 724 kg total N ha⁻¹ (Bittman et al., 2007). This response to manure application might not be entirely attributable to the provision of N to the crop by DMS. This study investigates the effect of nine years of DMS application on soil quality under tall fescue (*Festuca arundinacea* Schreb.). The renovation of the forage stand, and consequent interruption of DMS application the year prior to this study, allowed us to compare soil quality in systems receiving the same amount of mineral N as DMS or as ammonium nitrate (AN) without the confounding effect of residual release of N from last year DMS application.

6.3 Materials and Methods

This study was conducted at the Pacific Agri-Food Research Centre at Agassiz in south-coastal British Columbia (49° 10' north, 125° 15' west) to examine the long-term effects of multiple additions of DMS to grasses. The 3 x 65 m plots of tall fescue (*F. arundinacea* Scherb. var. *Festorina*) were established in 1993 on a Monroe (Deoric Regosol) silty to sandy loam soil with 6.3% organic matter and moderately good drainage (Bittman et al., 2007). Five treatments in a randomized complete block design were used: an unfertilized control, two dairy manure slurry treatments applied at target rates of 50 kg

ha⁻¹ (DMS₅₀) and 100 kg ha⁻¹ (DMS₁₀₀) of mineral-N, and two ammonium nitrate (AN) fertilizer treatments (AN₅₀ and AN₁₀₀) applied at the same rates of 50 kg ha⁻¹ and 100 kg ha⁻¹ mineral N. In addition inorganically treated plots also received 135 kg ha⁻¹ of K₂O, but no phosphorus was required. Since approximately 50% of total manure N was in organic form, the low N manure treatment received similar total N as the high N fertilizer treatment. The fertilization treatments were applied in early spring and after each harvest, except the last one, just before winter. In 2004, the sampling year, harvests were taken on 10 May, 5 July, 20 September and 12 November. The DMS was obtained from manure storages on local high-input dairy farm in which wood shavings are used for bedding. Manure was applied by surface-banding at 23-cm spacing with a 3-m wide sleigh-foot or drag-shoe slurry applicator mounted behind a 4,000-L tank. Phosphorus was applied to AN treated plots in spring as indicated by soil test. The tall fescue stands were restored through cultivation and reseeding in 2003, and the fertilizer treatments interrupted in 2003 were reinstated in 2004, in the same plots. The composition of the DMS used and the actual rates of application of slurry and fertilizers as well as other experimental details can be found elsewhere (Bittman et al., 2007). Average monthly temperature and total precipitation was recorded for the year 2004 (Table A.6)

6.3.1 Soil and plant sampling

The experimental soils were sampled three times during the growing season: on 15 June, 5 August, and 10 November of 2004. Soil samples were obtained from the top 7.5 cm of the soil using a standard hand soil probe 1 inch in diameter. Thirty soil cores were taken from each plots and pooled. After sieving through 2-mm sieve, each soil sample was divided into three portions. One was stored at -20°C for fatty acid methyl

ester analysis, the second was stored at 4°C for a short period before enzyme assay and microbial biomass carbon (MBC) determination and the third portion was air dried before analysis of the light fraction of soil organic matter (LFOM) as described below. Air dried subsamples were also used for total soil organic C (SOC) and N (SON) determination using NA1500 NCS Analyzer (Baccanti and Columbo, 1992).

A set of eight anion exchange membranes (AEM) (2.25 x 6.5 cm) was inserted into the soil of each plot, where pairs of strips were placed at four locations. The AEM were incubated in soil from (1) 8 June to 22 June, (2) 23 July to 20 August, (3) 4 October to 18 October, and (4) 19 October to 3 November of 2004. The membranes were removed from soil and washed with deionized water to remove the adhering soil before determination of soil P and NO₃ fluxes, as described below.

Plants were harvested from 1.5 x 7.6 m strips with a sickle bar mower. All of the herbage from the sampled area was weighed fresh in the field, then sub-sampled (0.7 kg or more) for determination of dry matter yield. Plants were weighed fresh and dried at 60°C until constant weight was recorded. The dried subsamples were ground in a Wiley mill through a 2-mm mesh screen. Ground plant samples were digested (Noel and Hambleton, 1976) and analyzed for tissue N and P concentrations by salicylate/nitroprusside, and acidic molybdate/ascorbic acid methods (Anonymous, 1976), respectively, on a Technicon AutoAnalyzer II.

6.3.2 Soil enzyme assay

The activities of three soil enzymes, dehydrogenase, phosphatase and urease, were assayed. Dehydrogenase was assayed by triphenyltetrazolium chloride (TTC) reduction, as reported by Casida et al. (1964). The phosphatase assay followed the

method of Eivazi and Tabatabai (1977). For the urease assay 2.5 g of soil were mixed with 1.3 mL of 0.08 N urea solution into a flask, capped and incubated at 37°C for 2 h. Then 25 mL of 1N KCl/ 0.1N HCl solution was added before shaking for 30 min. The suspension was passed through a Whatman No. 40 filter paper and analysed for $\text{NH}_4^+\text{-N}$ by the Kjeldahl method (Jones, 1984).

6.3.3 Microbial biomass

Soil MBC was determined using the soil fumigation extraction method (Voroney et al., 1993) using a 0.25 M K_2SO_4 extracting solution. Soil extracts were analyzed for organic carbon from both fumigated and non-fumigated samples using Tekmar-Dohrmann Phoenix 8000 Total Organic Carbon Analyzer (TOC-V). Soil microbial biomass was calculated using an extraction efficiency factor (K_{ec}) of 0.45 (Jenkinson et al., 2004).

6.3.4 Soil microbial community

Fatty acids in the phospholipid (PLFAs) and neutral lipid (NLFAs) fractions of soil extracts were analyzed and used as indicators of active soil microbial biomass and fungal storage material, respectively, using the method described in Chapter 4. Total soil lipids were extracted from 4 g of soil (dry weight equivalent) in dichloromethane (DCM): methanol (MeOH): citrate buffer (1:2:0.8 v/v). Identification of peaks was based on comparison of retention times to known standards (Supelco Bacterial Acid Methyl Esters #47080-U, plus MJS Biolynx #MT1208 for 16:1 ω 5). The abundance of individual PLFAs was expressed as $\mu\text{g PLFA g}^{-1}$ dry soil.

Individual fatty acids have been identified as signatures for various groups of microorganisms (Hamel et al., 2006; Pankhurst et al., 2002). The FAME 18:2, 18:1c and

18:1t were used as indicators of saprophytic fungal biomass (Petersen and Klug, 1994; Frostegård and Bååth, 1996) and FAME 16:1 ω 5, as indicator of arbuscular mycorrhizal fungi (Balser et al., 2005; Spring et al., 2000). FAME i-15:0, 15:0, 3OH-14:0, i-16:0, i-17:0 +17, 17:0, 18:0 and 2OH-16:0 were chosen to represent bacterial PLFAs (Jarvis and Tighe, 1994; Hamel et al., 2006;) based on the bacterial standards used while FAMES 16:0 is general biomarker (Spring et al., 2000).

6.3.5 Soil PO₄ and NO₃ flux determinations

Before placement in soil, anion exchange membranes strips were charged with bicarbonate anion by gentle shaking in 1 L 0.5M sodium bicarbonate overnight, followed by 3 rinses in deionized water. The strips were then kept in distilled water until soil placement. After recovery from the soil, membranes were extracted in 25 ml of 2 M KCl in 250 ml flasks by shaking for 1 h. The extracts were filtered. The P concentration in extract was determined colorimetrically by the molybdate-ascorbic acid method, and their NO₃-N concentration was measured by hydrazine reduction (Technicon AutoAnalyzer, 1976) using a Technicon segmented flow AutoAnalyzer (AAII system).

6.3.6 Light fraction of soil organic matter

The abundance of the LFOM was determined using a procedure reported by Strickland and Sollins (1987). Ten grams of air dry soil were suspended in 30 mL sodium iodide (NaI) of density 1.7 g cm⁻³ for 30 sec at 12000 rpm with a Virtis 23 mixer, and then left undisturbed for 48 h, before removal of the light fraction from the surface of the liquid using a vacuum apparatus with a Millipore filter. The light fraction was oven dried at 65°C and weighed. Soil samples were extracted twice following this procedure.

6.3.7 Statistical analysis

The significance of treatment effects on soil variables was assessed using repeated-measures analysis of variance (PROC MIXED) (Wang and Goonewardene, 2004) and that of plant related variables using ANOVA. The data were tested for normality using the Shapiro-Wilk's test. The LSD test was used for treatment means comparisons. The threshold used for rejection of null hypotheses was $\alpha = 0.05$. Regression analysis was used to compare plant growth response under DMS and AN. The yield data obtained the year of the study and data previously published from the same experiment (Bittman et al., 2007) were used. The published data consisted of average yields obtained with each treatment from 1996 to 2002. All statistical analyses were conducted with JMP 3.2.6 (SAS Institute, Cary, USA) except the correspondence analysis which was performed using SYSTAT 12. Correspondence analysis was conducted to define the impact of treatments on microbial community structure (Legendre and Legendre, 1998).

6.4 Results

6.4.1 Soil properties

Ten years of repeated additions of different rates of DMS and AN influenced soil properties. Total soil carbon was higher with DMS₁₀₀ than with DMS₅₀, AN₁₀₀, AN₅₀ and non-fertilized control (Table 6.1). Soil organic N content was also higher with DMS than with AN application or control (Table 6.1). The LFOM increased with increasing rates of manure application. High rate of mineral fertilizer application (AN₁₀₀) and control treatment were associated with similar amounts of LFOM, which were superior to those measured under AN₅₀ (Table 6.1). The addition of DMS and AN increased nitrate flux

Table 6.1 Soil (7.5 cm depth) properties as influenced by ten years of continuous application of DMS and mineral fertilizer to tall fescue.

Treatments/Parameters	TC (g kg ⁻¹)	TON (g kg ⁻¹)	LFOM (mg g ⁻¹)
Control	36.53 c	2.84 c	150 c
*AN ₅₀	36.75 c	2.88 c	149 d
AN ₁₀₀	35.85 c	2.92 c	151 c
DMS ₅₀	39.02 b	3.10 b	157 b
DMS ₁₀₀	41.82 a	3.30 a	161 a
S.E.	0.77	0.01	0.12

*AN₅₀ = 50 kg ha⁻¹ ammonium nitrate, AN₁₀₀ = 100 kg ha⁻¹ ammonium nitrate, DMS₅₀ = 50 kg ha⁻¹ dairy manure slurry, DMS₁₀₀ = 100 kg ha⁻¹ dairy manure slurry, TC = total soil carbon, TON = total organic nitrogen, LFOM = Light fraction of organic matter. S.E.: standard error of the means. Values following different letters within columns are significantly different according to protected Fisher's LSD test ($P = 0.05$, $n = 12$).

(AEM-NO₃) as compared to untreated control plots (Table 6.2). Nitrate fluxes were similar whether it was fertilized with DMS or AN at the same mineral N equivalent level, except for the 8-to-22 June period where fluxes were higher with DMS₁₀₀ than AN₁₀₀ (Table 6.2). Phosphate fluxes (AEM-PO₄) were highest under DMS, and lower under AN, sometimes significantly lower than under control treatment, and the differences widened with time in the season (Table 6.3).

6.4.2 Microbial biomass carbon and activity

Application of DMS₁₀₀ increased MBC as compared to other treatments (Table 6.4). Soil microbial activity, as assessed by dehydrogenase assay, was highest with both DMS application rates, and was reduced with AN₁₀₀ as compared to control (Table 6.4). The activity of urease followed a similar trend as found for dehydrogenase activity. It was enhanced by DMS₁₀₀ and reduced by AN₁₀₀ (Table 6.4). Phosphatase activity was not influenced by the treatments (Table A.7).

6.4.3 Microbial community structure

The soil microbial community structure evaluated by PLFA profiling varied with fertilization treatments. Correspondence analysis of PLFA biomarkers revealed stronger influence of AN and DMS on soil microbial community structure at higher rate of application (Fig. 6.1). The analysis showed that abundance of beneficial AMF biomarker 16:1 ω 5 and of several bacterial biomarkers (2OH-16:0, 18:0, 15:0, and i-16:0) was associated with DMS application. Scarcity of saprophytic fungi biomarkers was also associated with DMS application.

Table 6.2 Soil nitrate flux (AEM-NO₃) (µg cm⁻² d⁻¹) as influenced by ten years of continuous application of dairy manure and fertilizer during the growing season of tall fescue.

Treatment/Time	8 June	23 June	6 August	4 October	18 October
Control	0.21 d	0.00 c	0.03 b	1.36 c	1.92 b
*AN ₅₀	0.45 c	1.33 b	1.498 b	17.61 bc	4.28 b
AN ₁₀₀	4.60 a	3.74 a	5.11 a	37.69 a	17.72 a
DMS ₅₀	1.30 c	0.37 b	1.29 b	8.34 c	5.30 b
DMS ₁₀₀	2.63 b	4.00 a	6.40 a	29.62 ab	15.08 a
S.E.	0.37	0.67	1.09	5.56	1.49

*AN₅₀ = 50 kg ha⁻¹ ammonium nitrate, AN₁₀₀ = 100 kg ha⁻¹ ammonium nitrate, DMS₅₀ = 50 kg ha⁻¹ dairy manure slurry, DMS₁₀₀ = 100 kg ha⁻¹ dairy manure slurry. S.E.: standard error of means. The comparisons are made within the dates using protected Fisher's LSD ($P = 0.05$, $n = 4$).

Table 6.3 Soil phosphate flux (AEM-PO₄) (µg cm⁻² d⁻¹) as influenced by ten years of continuous application of dairy manure slurry and ammonium nitrate fertilizer to tall fescue during the growing season

Treatment/Time	8 June	23 June	6 August	4 October	18 October
Control	6.38 bc	4.55 ab	6.30 ns	1.67 c	1.23 c
*AN ₅₀	6.06 c	3.42 c	3.91 ns	0.67 c	0.35 d
AN ₁₀₀	6.07 c	3.42 c	3.91 ns	0.67 c	0.35 d
DMS ₅₀	7.43 a	4.52 ab	6.33 ns	4.48 b	2.47 b
DMS ₁₀₀	6.96 ab	5.46 a	7.72 ns	6.71 a	4.06 a
S.E.	0.30	0.45	0.94	0.51	0.29

AN₅₀ = 50 kg ha⁻¹ ammonium nitrate, AN₁₀₀ = 100 kg ha⁻¹ ammonium nitrate, DMS₅₀ = 50 kg ha⁻¹ dairy manure slurry, DMS₁₀₀ = 100 kg ha⁻¹ dairy manure slurry. S.E.: standard error of means. The comparisons are made within the dates using protected Fisher's LSD ($P = 0.05$, $n = 4$).

Table 6.4 Soil microbiological properties (MBC, dehydrogenase and urease) and plant nitrogen (N) and phosphorus (P) content as influenced by ten years of continuous application of DMS and mineral fertilizer to tall fescue.

Treatments/Parameters	MBC (mg kg ⁻¹)	Dehydrogenase (µg TPF g ⁻¹ d ⁻¹)	Urease (µg g ⁻¹)	Plant N (mg g ⁻¹)	Plant P (mg g ⁻¹)
Control	577.7 b	90.0 b	11.2 bc	18.4 c	4.4 a
*AN ₅₀	579.6 b	90.7 b	10.5 bc	20.8 b	3.3 c
AN ₁₀₀	562.6 b	67.0 c	10.1 c	27.7 a	3.0 d
DMS ₅₀	618.1 b	122.4 a	12.0 b	23.2 b	4.2 a
DMS ₁₀₀	671.6 a	111.5 a	12.2 a	27.0 a	4.0 b
S.E.	19.3	10.1	1.1	0.4	0.07

*AN₅₀ = 50 kg ha⁻¹ ammonium nitrate, AN₁₀₀ = 100 kg ha⁻¹ ammonium nitrate, DMS₅₀ = 50 kg ha⁻¹ dairy manure slurry, DMS₁₀₀ = 100 kg ha⁻¹ dairy manure slurry, MBC = microbial biomass carbon. S.E.: standard error of means. Values with different letters within columns are significantly different according to protected Fisher's LSD ($P = 0.05$, $n = 12$).

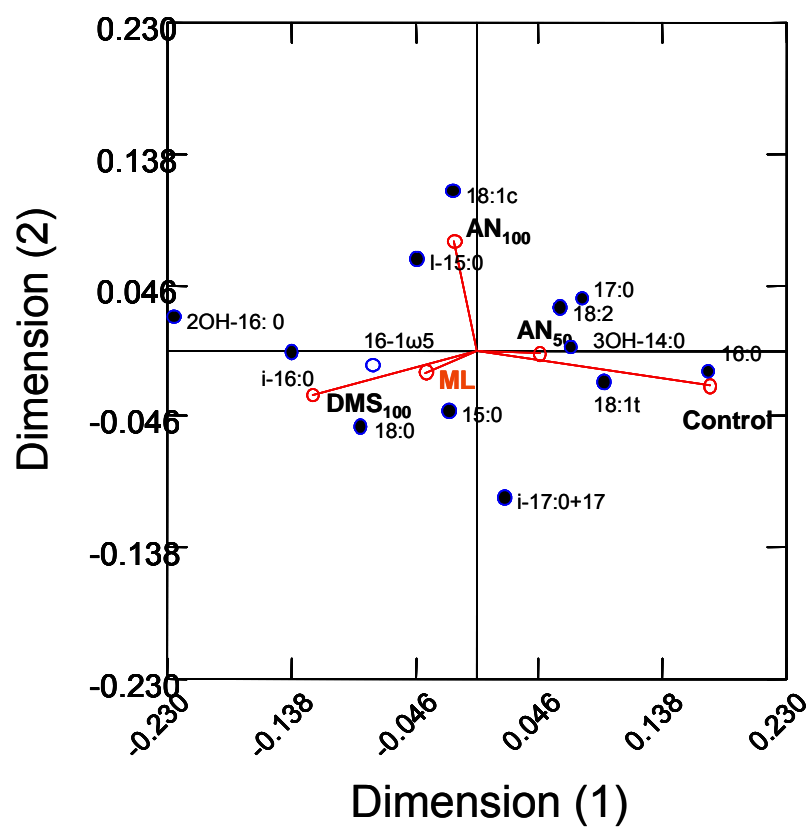


Figure 6.1 Correspondence analysis depicting relationship between PLFA biomarkers and dairy manure slurry and mineral fertilizers (N =20). AN₅₀ = 50 kg ha⁻¹ ammonium nitrate, AN₁₀₀ = 100 kg ha⁻¹ ammonium nitrate, DMS₅₀ = 50 kg ha⁻¹ dairy manure slurry, DMS₁₀₀ = 100 kg ha⁻¹ dairy manure slurry.

6.4.4 Plant biomass and nutrient concentration

A time x fertilization interaction indicated that the yield difference between the different fertilization treatments disappeared at the last harvest, when yields were very low (Fig. 6.2). The dry matter yield of tall fescue increased with the application of DMS and AN as compared to control, but similar yields were obtained with DMS and AN at all harvest times, when applied at the same mineral N equivalent level. The annual yield response to fertilization treatments was very similar under DMS and AN fertilization during the study season, which followed a year of plot restoration; this is in contrast with the different response curves obtained for tall fescue grown under DMS and AN before plots' renovation, as revealed by regression analysis of previous yield data (Fig. 6.3).

Independently of the type of fertilizer, plant N concentrations were similar at the same rate of application and, in this, followed a trend similar as that of yield. Plant P concentration was higher in DMS treatments as compared to AN treatments (Table 6.4).

6.5 Discussion

Our results show that DMS application not only improved soil N fertility but also soil productivity in other ways, which are most likely related to the stimulation of soil microorganisms. This increase in soil productivity is concurrent with increased risk of P loss to the environment.

6.5.1 Dairy manure slurry enhances the yield potential of the soil

The crop growth response curves calculated from the 1996 to 2002 yield data clearly showed an increase in the yield potential of the soil receiving DMS application prior to plant stand renovation. According to these curves, the yields achieved with DMS were not likely to be reached with additional amounts of AN, as the crop response had

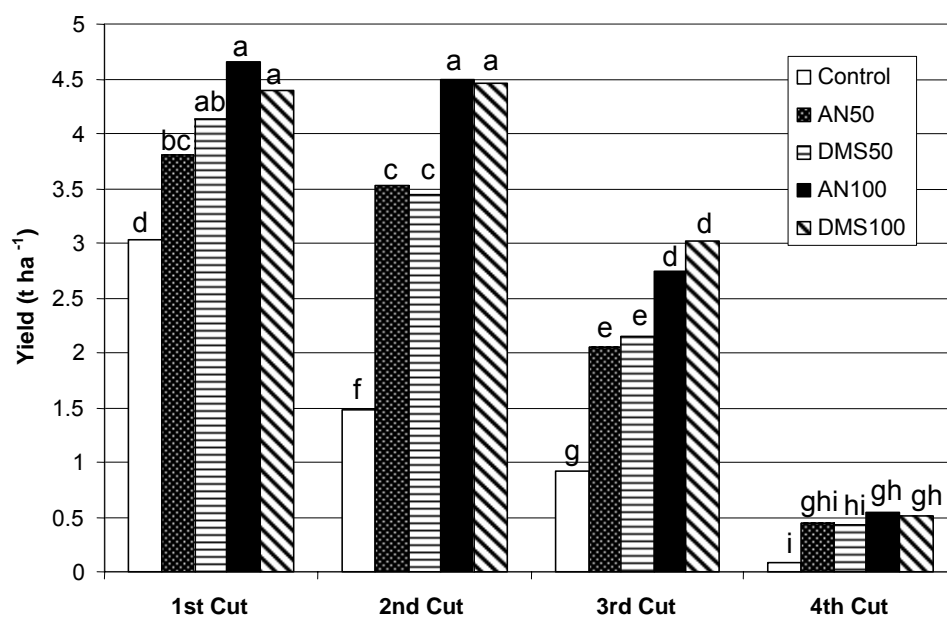


Figure 6.2 Tall fescue yield as influenced by DMS and mineral fertilizer application. Means associated with the same letter are not different according to protected Fisher's LSD test ($P = 0.05$, $n = 12$).

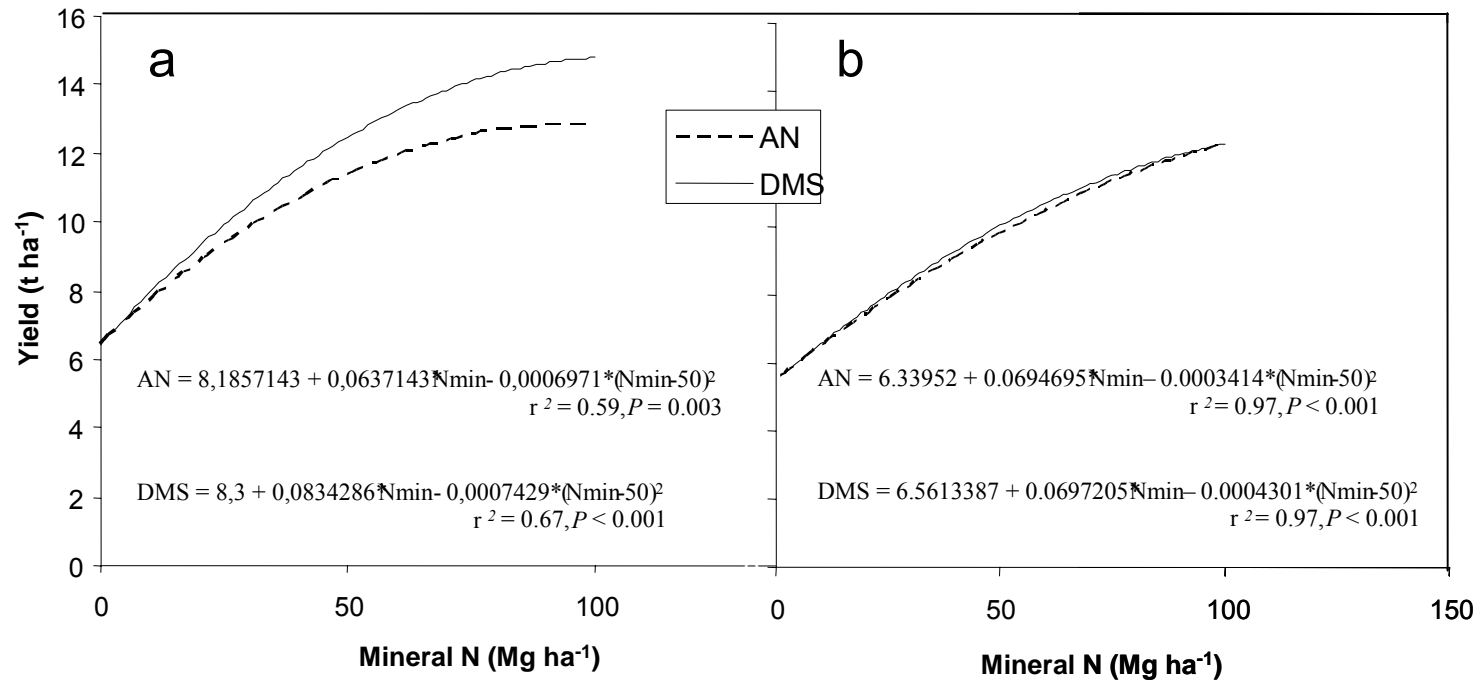


Figure 6.3 (a) Second degree polynomial yield response to ammonium nitrate (AN) and dairy manure slurry (DMS) application made on the basis of their mineral N content, derived from 1996-2002 mean annual herbage yield data (Bittman et al., 2007) and (b) yield response obtained in 2004, after restoration of the stands in 2003.

leveled off at the high AN rate. The DMS addition can influence soil fertility, physical properties and water relations (Tisdall and Oades, 1982; Hudson, 1994; Riffaldi et al., 1994) but how DMS increases soil productivity beyond the mere provision of plant available N in this precise case remains unclear, however. After a year of plant stand renovation without DMS application the N contribution of the organic fraction of DMS to the soil available N pool had disappeared, allowing for the examination of the other beneficial effects of DMS on soil quality. But, the difference between tall fescue yield fertilized with DMS and AN fertilization had also disappeared. We can only conclude that the factor or factors responsible for the greater productivity of the soil under DMS were eliminated by the disturbance created by stands renovation.

We measured positive effect of DMS application on important indicators of soil quality. The amount of soil organic matter, soil microbial biomass and activity, and nitrogen cycling were all increased in the DMS fertilized system, and this is in agreement with previous research results. Application of organic manure is known to increase soil organic matter content (Peacock et al., 2001; Martyniuk et al., 2002; Bohme et al., 2005; Fließbach et al., 2007; Yang et al., 2007). Soil organic matter content positively influence soil MBC (Birkhofer et al., 2008) and soil enzyme activity (Crecchio et al., 2001). Whether soil organic matter level and biological activity were higher did not seem to have any influence on yields in 2004, however. Instead, tall fescue yield was very dependant on the amount of mineral N applied to plant stands, as shown by crop growth response curves close to linearity and the high similarity of crop response to DMS and AN. Tall fescue yields produced under DMS and AN fertilization were remarkably similar when fertilized at the same rate of mineral N in 2004.

The disappearance of soil yield potential enhancement after the field was renovated and DMS application withdrawn for one year, in 2003, may be due to soil conditions different from those of published experiments reporting long lasting effect of manure application (Eghball et al., 2002; Mugwira et al., 2002; McAndrews et al., 2006). Application of manure to soils improves soil water retention capacity (Hudson, 1994), aggregate formation and stabilization (Tisdall and Oades, 1982), bulk density (Soane, 1990) and cation exchange capacity (Riffaldi et al., 1994), leading to increased plant productivity. In studies where improvement in crop yield with manure application has been reported, the soils were low in organic carbon content ranging from 1.8 to 4g kg⁻¹, while in our soils it was 60g kg⁻¹, suggesting that lasting crop response to manure application is more likely to occur in soils with low organic matter content. Soils of the Monroe series are friable, slightly acidic, with high cation exchange capacity. They offer a good rooting depth to crop plants and are conducive to biological activity (Luttmerding and Sprout, 1967). The soil at our study site contained over 6% of soil organic matter at the onset of the experiment in 1993. The addition of 4 g kg⁻¹ of organic C and 0.3 g g kg⁻¹ of organic N as measured under DMS₁₀₀, may not enhance further the properties of a soil already rich in soil organic matter. The fact that the soil had higher yield potential when managed under DMS fertilization does indicate, however, that the enhancing factor is not organic matter per se, but probably a consequence of it.

Addition of organic matter to soil with DMS application may have created soil conditions conducive to enhanced tall fescue productivity. Our results suggest that these conditions may have biological origin. Fertilization treatments had impacts on soil microbial biomass, community structure and enzyme activity. The possibility of a

biological origin of soil yield potential enhancement under DMS application is supported by previous research reporting a large influence of land use and crop management on soil microbial communities (Calderon et al., 2001; Steenwerth et al., 2003; Cruz et al., 2009; Meriles et al., 2009). The influence of crop management on soil productivity is the sum of many direct impacts and complex interactions between the numerous biotic and abiotic components of the soil system (Atul-Nayyar et al., 2008), which may take time to reach a new dynamic equilibrium. The conditions favouring tall fescue productivity under the DMS systems, which disappeared with stand renovation, seems to require more than one growing season for re-establishment. The influence of management on the soil systems is sometimes slow to develop (Chan et al., 2003). For example, it takes about five years of crop influence for the development of the biological and chemical soil conditions conducive to Fusarium crown and root rot of asparagus (Hamel et al., 2005). The recovery of soil structure after tillage is also a lengthy process. Changes in soil pores quality occurring over more than four years after tillage has ceased was identified as a main factor explaining reduced yields in soils recently converted into no-till (VandenBygaart et al., 1999). These soils are characterized by horizontally oriented elongated macropores, limiting drainage and root penetration. Rounded macropores increase with the number of consecutive years in no-till as these pores are maintained each year due to the lack of tillage and greater faunal activity creates more of these pores (Six et al., 2004).

6.5.2 Dairy manure slurry increases phosphate flux in soil

Besides N, animal manure is also a good source of P. Management of animal manures to provide plant nutrients is often based on crop N requirement. However, N

based manure applications supply crop-soil system with excess P increasing off-field P movement (Toth et al., 2006). Ten years of DMS application at the study site resulted in phosphate fluxes more than twice as large as found in the system fertilized with mineral fertilizer, indicating higher level of saturation of soil P fixation capacity. At the tissue P level and yield measured in 2004, 59.2 kg ha⁻¹ of P was exported in hay yield as compared to about 549 kg ha⁻¹ of P added in the DMS₁₀₀ fertilization system. As the amount of P in soils increases with repeated excessive P application while fertilizing crops based on their N need, the risk of P losses through surface run off and leaching increases along with the risk of eutrophication in recipient surface water bodies (Sharpley, 1996). This risk remains to be evaluated in the Fraser River Valley as the negative effect of DMS on soil P loading must be considered along with its beneficial effect on soil productivity, by land users and policy makers.

6.6 Conclusion

There is no simple definition of soil quality. We found that DMS application efficiently improves the status of most soil quality indicators, including yield potential. This yield potential enhancement appears to be of biological origin as it was eliminated by disturbance related to tall fescue stand renovation and apparently requires more than one growing season to be re-established. While DMS enhances soil productivity, the application of P in excess of crop needs with continuous application of DMS to satisfy tall fescue N need leads to increase in soil P flux. Thus, it appears that fertilization with DMS enhances soil productivity, arguably the most important function of an agricultural soil, but this practice may also increase the risk of P loss from agriculture.

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7. Preface

The following section presents an analysis of the combined data. It reveals an effect of N and P inputs on soil microbial community structure and AMF abundance that is consistent throughout soil and climatic conditions. Different agroecosystems with different environmental conditions exhibited different soil nutrient status. Soil nutrient status influences soil microbial community structure.

7. GENERAL DISCUSSION

Soil microorganisms are responsible for many functions such as soil organic matter turnover, soil humus formation, cycling of nutrients and building soil structure. These functions are performed by many different genera and species of microorganisms, and soil microbial diversity is critical to soil functioning. However, this microbial biodiversity is influenced by crop and soil management practices. My research assessed the effect of long-term application of P, N, organic C and crop type on the soil microbial community and plant-AMF interactions in different agroecosystems.

7.1 Fertilizer Input and Microorganisms

Bacterial and fungal communities were influenced by the soil nutrient status. The microbial PLFA, soil NO₃ and PO₄ fluxes and soil organic C data of the studies reported in Chapters 4, 5 and 6 were obtained using the same protocols. These data were combined into a large dataset and subjected to canonical correspondence analysis (CCA) to assess the effect of soil N and P fertility and soil organic C on soil microbial community structure using PC-ORD, version 4.34 (MjM Software, Gleneden Beach, OR). The CCA revealed a strong relationship (Fig 7.1, $P = 0.02$) between the soil microbial community and soil fertility status. High soil NO₃ fluxes measured in systems fertilized with manure (Chapter 6) or mineral N (Chapter 5) were associated with peak abundance of certain bacterial PLFA biomarkers (3OH-12:0, a-15:0, 18:0, 18:1t), two non-specific eukaryotic biomarkers also present in plants (16:0, 14:0) and the biomarker for saprophytic fungi (18:2). Soil P and organic C seemed to favour bacteria (3OH-14:0,

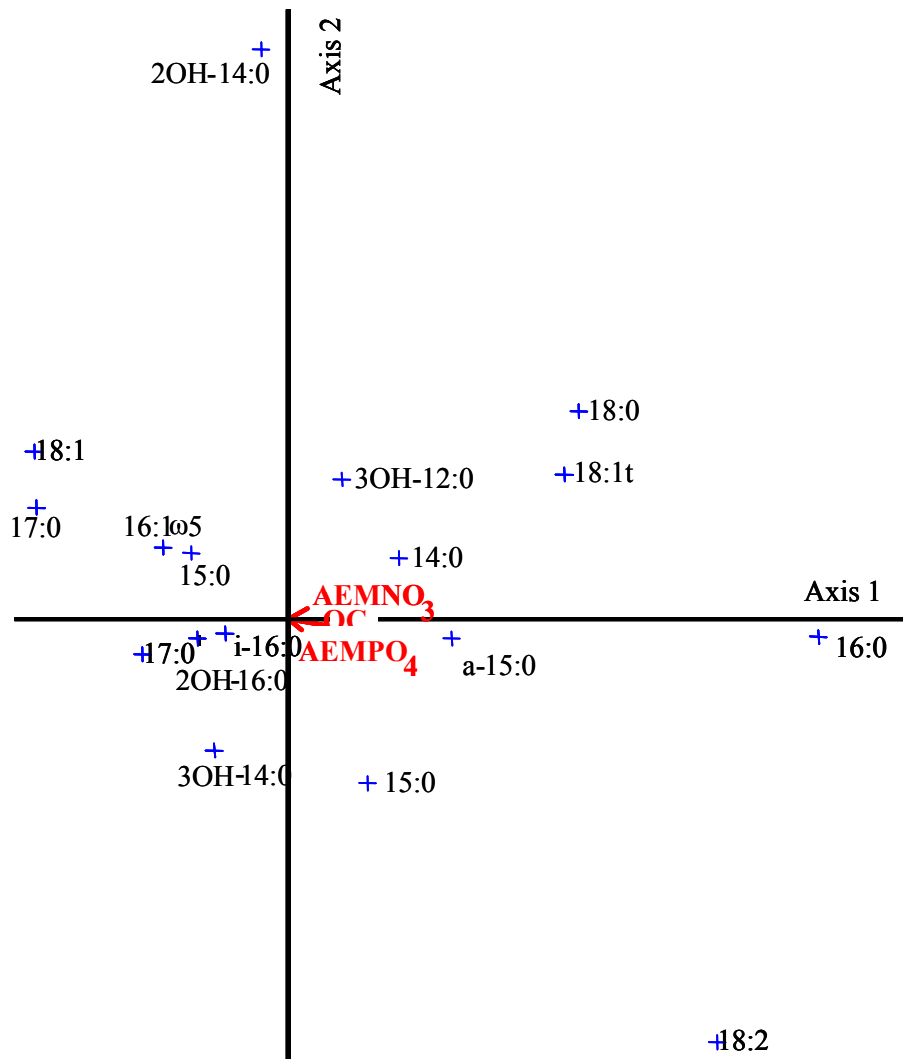


Figure 7.1 Canonical; correspondence analysis (CCA) ordination plot ($P = 0.02$) of the relationships between PLFA biomarkers (cross) and the soil parameters (AEMNO₃, nitrate flux; AEMPO₄, phosphate flux; OC, organic carbon level) for different agroecosystems.

a-15:0, i-15:0) (Fig. 7.1). Saprophytic fungi (18:2) were highly related to soil C and P fertility. The size of bacterial population represented by PLFA biomarkers 18:1, i-17:0, 15:0, 2OH-16:0, i-16:0, 17:0 and 3OH-14:0 and AMF (16:1 ω 5) peaked at lower soil nutrient status (Fig. 7.1). These results suggest that the abundance of soil microorganisms depends on soil nutrient status and that the population of soil microbes in different agroecosystems will vary with soil nutrient availability. The fact that the abundance of the general biomarker 16:0 peaks at high NO₃ fluxes suggests that the effect of N fertilization on the abundance of soil microorganisms is associated with plant productivity. Other studies have also reported that addition of manure may influence the soil microbial community and biological activity in soils (Bossio et al., 1998; Carpenter-Boggs et al., 2000).

Application of manure at the Agassiz site provided C but also N and P. Treatments applied at the Swift Current and Indian Head sites resulted in different plant productivity level, hence, different levels of C input to soil. The three experimental sites thus varied in N and P fertility and C input. The abundance of the non-specific eukaryotic PLFA biomarker 16:0 at high soil N and P fertility and organic C level suggests that higher plant biomass production in nutrient rich soils may be one cause of the abundance of some specific bacterial and fungal groups. Individuals of these groups could be rhizospheric, or more likely r-strategists which have their maximum intrinsic growth rate when resources are abundant (Bastian et al., 2009).

The changes in the soil microbial community due to fertilization can subsequently affect nutrient cycling and soil functioning. Since ecosystem functioning is governed largely by soil microbial dynamics, soil management practices affecting the soil

microbial community and activity may influence soil functioning (Balota et al., 2004). Thus, it is important to understand the effect of soil management practices on the soil microbial community and activity. A relationship between the soil microbial community structure and soil enzymes from all experimental sites was revealed by CCA analysis (Fig. 7.2). This analysis was calculated after pooling the data from studies reported in Chapters 4, 5 and 6, as described above. It also revealed that the activity of different enzymes was associated with different broad taxonomic groups of microorganisms. Greater bacterial biomass was associated with high biological activity, as revealed with dehydrogenase activity; saprophytic fungi were associated with urease activity, whereas AMF biomass showed a higher association with P demand, as expressed by phosphatase activity (Fig. 7.2). These results suggest that microbial activity (as represented by enzyme assays), and thus soil functioning, varied under different soil conditions. This study reveals that long-term N, P and manure input and cropping system influence soil quality and functioning.

7.2 Fertilizer Input and AMF

Arbuscular mycorrhizal fungi (AMF) are the largest component of the soil fungal community (Gosling et al., 2006). They facilitate plant uptake of mineral nutrients, especially immobile nutrients such as P, Cu, Zn (Ryan et al., 2008), and their role in N uptake has been demonstrated (Govindarajulu et al., 2005). Besides their effects on plant nutrition, AMF also play an important role in modulation of plant resistance to pathogens (Elsen et al., 2008), water and salt stress (Miransari et al., 2008), and in improving soil structure through exudation of glomalin (Wu et al., 2008).

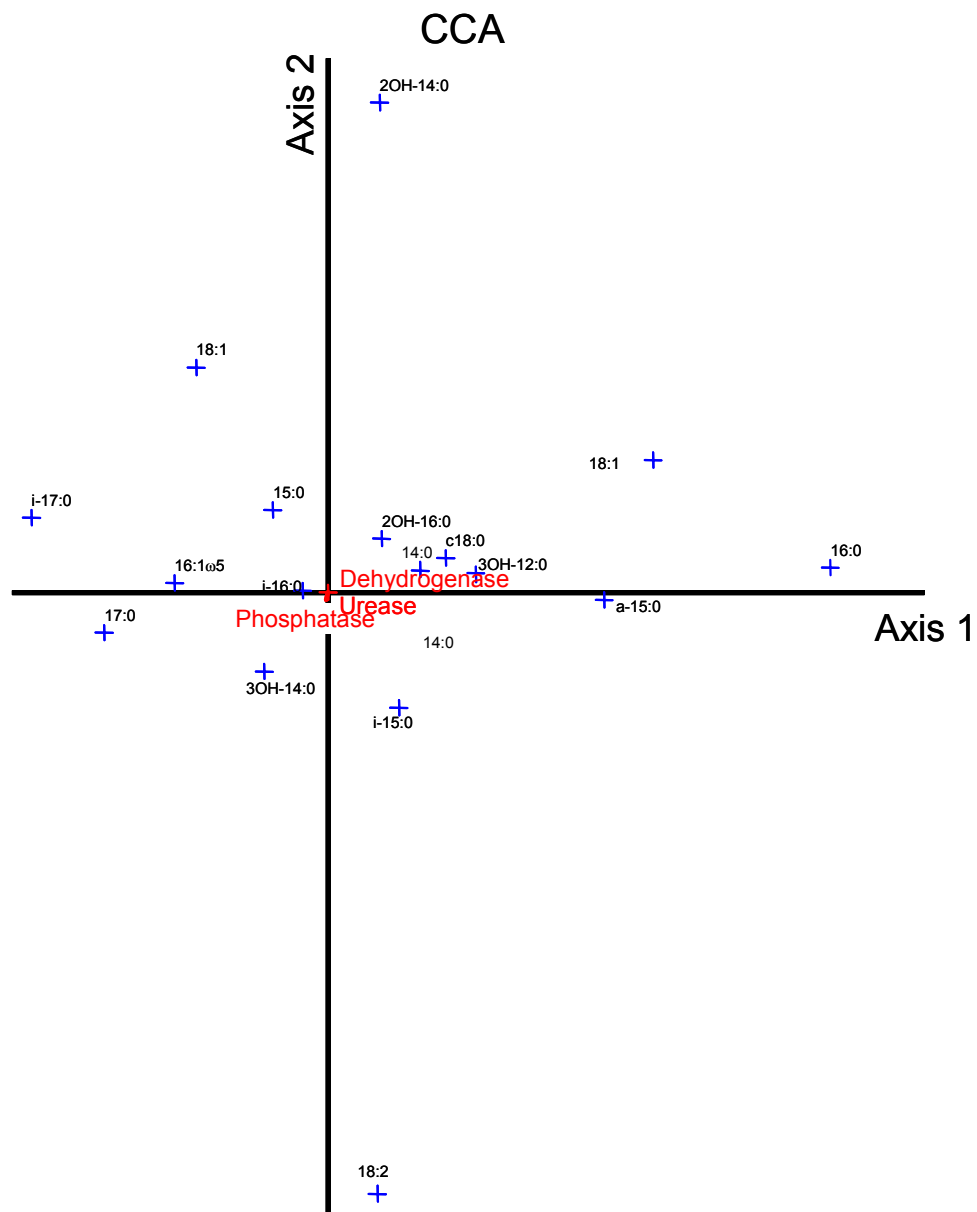


Figure 7.2 Canonical correspondence analysis (CCA) ordination plot ($P = 0.04$) of the relationships between PLFA biomarkers (cross) and the soil enzymes for different agroecosystems.

My greenhouse study revealed that AMF can act as a cornerstone of soil function and nutrient cycling (Atul-Nayyar et al., 2009). Although AMF have evolved as beneficial to host plants, their functionality may be influenced by changes in the soil environment related to soil fertility management in agroecosystems.

The field studies included in my thesis show that application of N, P and organic C-containing fertilizers influence plant-AMF interactions in different agroecosystems. High N, P and organic C levels in soil negatively impact the abundance of AMF (Covacevich et al., 2007; Treseder, 2004). My data show that AMF (PLFA 16:1 ω 5) were more distributed in soils with relatively low PO₄ flux levels, and relatively low NO₃ and organic C. The AMF are reported to proliferate in organic matter and scavenge the mineral N released from soil organic particles (Hamel, 2004; St. John et al., 1983; Chapter 3). The hyphae of AMF can also take up amino acids (Govindarajulu et al., 2005; Hawkins et al., 2000). Based on the observation of hyphae and vesicles of AMF in decomposing leaves of *Myrica parvifolia*, *M. pubescens* and *Paepalanthus* sp., Aristizábal and collaborators (2004) proposed that AMF proliferation accelerates the decomposition of organic matter and helps to efficiently recycle the mineral nutrients released by microbial decomposers. However, manure contains high amount of N and P which could repress AMF proliferation. It seems that lack of response of AMF to manure application at the Agassiz site could be due to high amounts of N and P present in manure mitigating the positive effect of organic matter on AMF proliferation. It is recognized that the impact of mineral fertilizer application usually decreases the density of AMF spores and hyphae, but only a few studies have reported their long-term effects on AMF extraradical development (Gryndler et al., 2006; Wilson et al., 2009). The results of the

study demonstrate that long-term application of either mineral or organic forms of N or P decreases AMF root colonization (Chapters 4, 5 and 6) and extraradical proliferation as revealed by the abundance of PLFA 16:1 ω 5 (Fig 7.1). This is consistent with some other long-term experiments. For example, Gryndler et al. (2006) observed less AMF extraradical proliferation after 51 years of elevated mineral or organic fertilization regime. Wilson et al. (2009), in contrasts, report increased extraradical AMF development after 23 years of annual nitrate application (100kg ha⁻¹). Kahiluoto et al. (2001) observed decreases in AMF densities under different soil and climate conditions upon application of more than 50 kg P ha⁻¹. In another study, several years of N application in the form of NH₄NO₃ at levels between 100 and 170 kg N ha⁻¹ yr⁻¹ decreased AMF spore densities in four North American grassland soils (Johnson et al., 2003). My study suggests that a high soil fertility level can reduce the abundance of AMF in cultivated soils, and thus modify plant-AMF interactions. The role of AMF in crop production is likely larger in low input agroecosystems.

7.3 Crop Type and Microorganisms

Crop rotation is important to maintain and improve soil quality, to enhance nutrient and water use efficiency, to improve N inputs through biological N fixation in legume based rotations and to break the life cycles of plant pathogens (Haramoto and Gallandt, 2004; Watson et al., 2002). Among biotic and abiotic factors influencing the biodiversity of AMF, host plant is the most important (Wang et al., 2005). Not all the plants that form symbioses with AMF show positive response to colonization. Arbuscular mycorrhizal fungi can sometimes be harmful to plants, depending on the plant and soil conditions (Douglas, 2008; Li et al., 2008).

My research shows that the plant-AMF interaction can vary from symbiotic to parasitic in different agroecosystems. A negative interaction between plant-AMF was observed with introduction of Russian wildrye (RWR) in alfalfa-RWR dual culture (Chapter 4). Russian wildrye supported higher levels of fungivorous and omnivorous nematodes which impaired the function of AMF symbiosis through fragmentation of the AMF hyphal network in soil. The C drain created by nematodes feeding on AMF led to reduced plant productivity (Li et al., 2008). In contrast to the alfalfa-RWR system, diversifications of pea-based rotation led to an increase in AMF abundance (PLFA) as compared to a pea monocrop (Chapter 5). The higher abundance of AMF in the pea-wheat system as compared to continuous-pea was likely due to the lower levels of soil N and an increased amount of organic matter production and input to soil observed for this rotation. Cruz et al. (2004) also reported improved AMF under low levels of soil N. The greater abundance of AMF in the pea-wheat rotation could have resulted in a reduced incidence of root rot thereby enhancing crop performance in the pea-wheat rotation as compared to the pea monocrop. The role of AMF in disease suppression is well known (Akhtar and Siddiqui, 2007; Lioussanne et al., 2007).

The role of the arbuscular mycorrhizal symbiosis can be important in agroecosystems in which organic nutrient sources such as N fixation replaces mineral fertilizers. Mäder et al. (2000) reported that under identical crop rotation and tillage scheme, arbuscular mycorrhizal root colonization was higher with organic input than conventionally farmed soils. However, in the present research work, arbuscular mycorrhizal root colonization was similar with application of dairy manure slurry (DMS) and mineral N. The differences in results in the two studies could be due to the

differences in amount of available N added into soil through mineral or organic sources. The amount of available N added through mineral N was larger than that added through (FYM) and slurry in the study conducted by Mäder et al. (2000). However, the amount of available N added through DMS and mineral N was the same in this study suggesting that high levels of soil available N represses arbuscular mycorrhizal development irrespective of N applied as mineral or organic fertilizer.

7.4 Conclusion

This Ph.D. thesis reports on how soil microbial communities and plant-AMF interactions in agroecosystems are influenced by soil fertility management and the choice of crops. Higher levels of soil nutrients select for certain bacterial groups, while AMF and other bacteria are more abundant under low input agroecosystems. The contribution of arbuscular mycorrhizal symbiosis to plant growth varied with the crop, from being negative to positive. The next step toward soil bioresource management might be to use management practices to manipulate soil microbial activity and enhance crop productivity.

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APPENDIX A

Table A.1 Microbial biomass as represented by total microbial biomarker in different AMF treatments.

Species	Total PLFA biomarker ($\mu\text{g g}^{-1}$)
Control	63.31 a
<i>G. claroideum</i>	66.32 a
<i>G. clarum</i>	30.93 a
<i>G. intraradices</i>	30.43 a
No significant effect was detected by ANOVA ($P = 0.05$)	

Table A.2 Average monthly temperature and total precipitation for Swift Current, Saskatchewan for year 2004

Month	Average temperature (°C)	Total precipitation (mm)
Jan	-14.3	37.6
Feb	-8	14.2
Mar	-1.2	12.6
Apr	6.3	10
May	8.6	83.7
Jun	12.9	66.2
Jul	17.6	61.1
Aug	15.3	72.3
Sep	12	27.1
Oct	4.5	20.8
Nov	-0.5	4.3
Dec	-7.4	27.3

Table A.3 Soil moisture content at different level of phosphorus fertilization

Phosphorus levels	Moisture (%)
0	9.24 a
20	8.93 a
40	8.93 a
SE	0.68

No significant effect was detected by ANOVA ($P = 0.05$)

Table A.4 Soil moisture content in monoculture and dual culture

Crop	Moisture (%)
Monoculture	8.89 a
Dual Culture	9.18 a
SE	0.55

No significant effect was detected by ANOVA ($P = 0.05$)

Table A.5 Average monthly temperature and total precipitation for Indian Head, Saskatchewan for the year 2005

Month	Average temperature (°C)	Total precipitation (mm)
Jan	-18.3	37.0
Feb	-11.9	6.8
Mar	-6.6	29.0
Apr	5.5	6.8
May	8.7	57.6
Jun	14.8	99.2
Jul	16.9	59.2
Aug	15.6	98.0
Sep	12.0	4.0
Oct	4.4	6.6
Nov	-3.8	34.9
Dec	-9.6	24.7

Table A.6 Average monthly temperature and total precipitation for Agassiz, British Columbia for the year 2004

Month	Average temperature (°C)	Total precipitation (mm)
Jan	2.8	164.5
Feb	6.5	59.8
Mar	8.6	205.4
Apr	12.5	22
May	14.2	100.4
Jun	17.6	38.8
Jul	20.0	19.0
Aug	19.8	115.4
Sep	14.6	166.8
Oct	11.3	173
Nov	5.9	304.8
Dec	5.2	224.5

Table A.7 Phosphatase activity in manure and inorganic fertilizer treated plots

Treatments	Phosphatase $\mu\text{g p-nitrophenol produced g}^{-1} \text{h}^{-1}$
Control	57.07 a
AN ₅₀	65.38 a
AN ₁₀₀	57.64 a
DMS ₅₀	61.13 a
DMS ₁₀₀	63.28 a