The Effect of Preceding Crop on Soybean Grain Yield, Mycorrhizal Colonization and Biological Nitrogen Fixation

BY

DON SANDERS

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree of

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Department of Plant Science

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FACULTY OF GRADUATE STUDIES

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TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Sanders, Donald W. The University of Manitoba, March, 2017. The effect of preceding crop on soybean (*Glycine max*) grain yield, mycorrhizal colonization, and biological nitrogen fixation. Major Professor:Yvonne Lawley.

Manitoba has seen a twenty-fold increase in soybean acres seeded since 2000, with over 1.6 million acres seeded in 2016. This change presents unique opportunities and challenges to improve crop rotations in Manitoba. This experiment studied the effect of four crop sequences on soybean yield, mycorrhizal colonization, and biological nitrogen fixation. In the first year of this experiment, spring wheat, canola, corn and soybeans were grown at three sites in Manitoba (Carman, Portage la Prairie, and Kelburn). In the second year, soybeans were grown on these same plots as a test crop. This two-year sequence of crops was done twice at each site, in 2012-13 and 2013-14. To determine mycorrhizal colonization, root samples were collected at the V3 stage and then analyzed microscopically for mycorrhizal infection. Nitrogen fixation was estimated using the natural abundance method using soybeans collected at the R5 and R6 stage and canola as a reference crop. Soybean following soybean had significantly higher grain yield than all other crop sequences at one site year, and significantly lower grain yield than all other crop sequences at another site year. There were no other differences in soybean test crop yield between crop sequences. Crop sequence significantly affected mycorrhizal colonization. Soybean following canola had significantly lower mycorrhizal colonization than soybean following soybean or corn. Soybean following spring wheat also had

significantly lower mycorrhizal colonization than soybean following soybean or corn. Soil test phosphorus levels also significantly affected mycorrhizal colonization, with increasing soil phosphorus resulting in decreased mycorrhizal colonization. Crop sequence significantly affected biological nitrogen fixation. Soybean following soybean or corn often had significantly greater biological nitrogen fixation than soybean following spring wheat or canola. Soil test nitrate levels affected biological nitrogen fixation, with increasing soil nitrate resulting in decreased biological nitrogen fixation. Soil test nitrate levels were affected by the carbon to nitrogen ratio of the preceding crop, with a higher carbon to nitrogen ratio associated with decreased soil nitrate. These results indicate that although there is often not a yield penalty associated with specific rotations, crop sequence has a strong impact on mycorrhizal colonization and biological nitrogen fixation. The soil organisms associated with those processes affect soil phosphorus uptake and nitrogen fixation. Producers should consider the importance of crop rotation when seeking to maximize productivity through symbiotic processes with mycorrhizae and nodule forming bacteria.

1.0 INTRODUCTION

Soybean production has increased exponentially in Manitoba in the last twenty years. The advent of early maturing, high yielding varieties has allowed soybean production to become profitable in Manitoba's growing conditions and climate. The wide variety of uses for soybean products and the large export market available to Manitoba growers offers the potential for a profitable crop. The ability of soybean to biologically fix nitrogen via *Bradyrhizobium* bacteria in root nodules also offers producers an economic and agronomic advantage in terms of fertilizer need and soil fertility. The question of how best to integrate soybeans into current Manitoba crop rotations offers unique opportunities and challenges to growers.

Crop rotation strategies for traditional soybean growing areas in North America have largely consisted of rotating soybean with corn. While this two-crop rotation results in higher yields, greater profits and improved soil characteristics over a simple monoculture, the lack of diversity can result in a number of challenges. Compared to more diverse rotations, a two-crop rotation can result in decreased yields, increased incidence of disease and pests, and declining soil physical properties (Bullock, 1992). Although crop rotations in Manitoba rely heavily on wheat and canola, options to market other crops in Manitoba allow producers the opportunity for a more diverse crop rotation than the corn-soybean rotation commonly found in the Midwestern United States. Incorporating soybean into Manitoba crop rotations in a method that results in high yields and improved agronomic outcomes is a goal that will result in continued research on soybean in Manitoba.

Soybean forms symbiotic relationships with both arbuscular mycorrhizal fungi (AMF) and *Bradyrhizobium* nodule forming bacteria, primarily *B. japonicum*. Both organisms provide the plant with nutrients in exchange for carbohydrates. AMF provide the plant with phosphorus as well as other nutrients, while *Bradyrhizobium* biologically fix nitrogen from the atmosphere, producing NH_4^+ that is useful to the plant. Both these sources are an important part of nutrient acquisition for the soybean plant. Crop rotation may affect the degree of colonization by these organisms which in turn may affect nutrient uptake and plant yield (Douds et al., 1997; Ferreira et al., 2000; Karasawa et al., 2002; Chen et al., 2013).

The goal of the following research was to quantify the effect that crop rotation has on soybean yield, colonization of the root system by AMF, and amount of biological N fixation. The objectives of this research were to determine:

i) the effect of crop rotation on soybean grain yield.

ii) the effect of crop rotation on the degree of colonization by AMF.

iii) the effect of crop rotation on the amount of N in the plant derived from biological N fixation.

iv) if the degree of mycorrhizal colonization in the plant has an effect on the amount of biological N fixation.

2.0 LITERATURE REVIEW

2.1 Introduction

Crop rotation is defined as the practice of growing different crops on the same area of land over multiple growing seasons (Yates, 1954). This is in contrast to continuous cropping, wherein the same crop is grown on the same area of land year after year (Power, 1990). It has been an important tool in crop production for thousands of years (Bullock, 1992). Crop rotation affects many aspects of crop production; yield, soil fertility, disease pressure, residue management, mycorrhizal colonization and biological nitrogen fixation (BNF) are some factors that are affected by rotation (Bullock, 1992). As producers in Manitoba dedicate more acres to soybean production, crop rotation will play an important role in their management decisions. This literature review will focus on the effects of crop rotation on yield, mycorrhizal colonization, and biological nitrogen fixation in soybean as these are of special interest to optimize soybean production in Manitoba.

2.1.1 The History of Soybean

The soybean plant (*Glycine max (L.) Merrill*) is a tropical legume that originated from East Asia, and was domesticated between 5000 and 9000 years ago in China (Lee et al., 2011). Soybean was also domesticated independently in Japan between 5000 and 7000 years ago, as well as Korea about 3000 years ago (Lee et al., 2011). Soybean was initially introduced to North America in 1765, and to Europe, South America, and Australia throughout the 19th century (Chaplin, 1996). Soybean was grown as early as 1855 in Canada, and by 1898 soybean was cultivated in Manitoba (Shurtleff and Aoyagi, 2010). For many years, soybean was mainly used as a forage crop in North America until

the early twentieth century, when other uses for the crop were discovered and production began to expand rapidly (Shurtleff and Aoyagi, 2010). Acres seeded in Manitoba were relatively low in the 20th century, with only 800 acres seeded in 1996. With the introduction of early maturing varieties that made soybean production viable in Manitoba, acres dedicated to soybean increased rapidly from 2000 onwards (Statistics Canada, 2015). In 2001, over 45 000 acres were seeded to soybean. This increased to 1.3 million acres in 2015 (Statistics Canada, 2015).

2.1.2 Soybean Production Statistics

Soybean is one of the most important food sources in the world. Global soybean production reached 319 million metric tonnes in 2015, with the top producers being the United States, Brazil, Argentina, China and India (United States Department of Agriculture, 2016). Soybean is the most produced grain oilseed worldwide (United States Department of Agriculture, 2016). Soybean production has been rising consistently in both Canada and Manitoba for the past 15 years. In 2015, Canadian soybean production reached a high of 6.2 million tonnes despite a 2.2 percent decrease in harvested area (Statistics Canada, 2015). Average yield in Canada was 42.4 bushels per acre (Statistics Canada, 2015). In 2015, Manitoba produced 1.3 million tonnes of soybean grain on 1.3 million acres of land (Statistics Canada, 2015). Soybean yield in Manitoba averaged 35.2 bushels per acre in 2015, 7.2 bushels lower than average yield in Canada (Statistics Canada, 2015). The shorter growing season in Manitoba compared to other soybean producing parts of Canada as well as the early maturing varieties planted results in lower yield than the Canadian average (Dorff, 2009).

2.2 Crop Rotation

2.2.1 Introduction

Crop rotations are designed to increase yield, profit, soil fertility and sustainability of the agroecosystem. Crop rotation provides a number of agronomic benefits, including improvements to: nutrient supply (Bolton et al., 1976; Kurtz et al., 1984; Power, 1990), soil moisture (Roder et al., 1989), soil structure (Griffith et al., 1988), insect and weed pressure (Bhowmik and Doll, 1982; Bullock, 1992), disease pressure (Dabney et al., 1988; Bullock, 1992), soil microbes (Williams and Schmitthenner, 1962) and crop residue breakdown (McDaniel et al., 2014). Crop rotation can also provide management benefits to growers such as improved timeliness of seeding and harvesting (Roth, 1996). Rotation can also reduce the need for tillage compared to rotations that produce high quantities of residue such as continuous corn (Morrison, 2013) Poor crop rotations may lead to detrimental economic and agronomic outcomes.

Crop rotations vary greatly in length and complexity. Crop rotations may rotate between different plant types, such as broadleaves and grasses. Rotations may also rotate between plants with different life cycles, such as annuals, perennials, biennials and winter annuals. Rotations may vary in length from short rotations consisting of two growing seasons to very long and complex rotations consisting of dozens of growing seasons (Bullock, 1992). Crop rotations may also incorporate fallow periods where no crop is grown (Bullock, 1992). A common example of a simple crop rotation is the corn-soybean rotation that dominates the Mid-Western United States (Bullock, 1992). Rotations can become increasingly complex, such as those practiced in Argentina that

take 10 to 12 years and involve four to six years of continuous grass-legume pasture followed by six years of cash grains (Bullock, 1992).

Crop rotations have become shorter in North America over the past fifty years (Bullock, 1992). Commercial fertilizers that replaced nitrogen and phosphorus removed with the grain allowed producers to exclude nitrogen-fixing legumes from their rotations without depleting soil nutrients (Bullock, 1992). Pesticides that reduced weed, insect and disease pressure also contributed to the shift to shorter rotations (Bullock, 1992). The cost of equipment and time management considerations also make extended rotations difficult or impractical for some producers (Bullock, 1992). In North America, the shift to shorter crop rotations in the last fifty years has led to decreased soil organic matter, degraded soil characteristics such as soil aggregate stability, bulk density, water infiltration rate and soil erosion, and increased external inputs (Bullock, 1992).

Crop rotations on the Canadian Prairies can vary greatly in complexity, but have been generally decreasing in complexity with the wide spread use and adoption of affordable commercial fertilizers and pesticides (Bullock, 1992). Spring wheat (*Triticum aestivum L.*) rotated with summer fallow was the most common rotation on the Canadian prairies during the late nineteenth century and most of the twentieth century (Campbell et al., 1990). Summer fallow acres declined as no-till cropping allowed producers to conserve moisture in dry areas and long-term research showed that annual grain production is higher in continuously cropped rotations. As acres dedicated to canola (*Brassica napus L.*) production increased dramatically with the introduction of herbicide tolerance traits in the 1990's, wheat rotated with canola has become the most popular crop rotation on the prairies (Kubinec, 2012)

2.2.2 Crop Rotation in Manitoba

Wheat and canola are the most commonly grown crops in Manitoba (Statistics Canada, 2015). In 2015, 4.2 million metric tonnes of wheat were harvested on 1.2 million ha of land in Manitoba (Statistics Canada, 2015). In 2015, 2.9 million tonnes of canola were harvested over 1.3 million ha of land. In 2015, soybean represented the third most seeded acreage in Manitoba, with 1.4 million tonnes harvested over 530 000 ha of land. Other crops commonly grown in Manitoba include corn (*Zea mays L.*) for grain (790 000 tonnes), oats (*Avena sativa L.*) (600 000 tonnes), barley (*Hordeum vulgare L.*) (570 000 tonnes), dry beans (*Phaseolus vulgaris L.*) (81 000 tonnes), rye (*Secale cereal L.*) (79 000 tonnes), peas (*Pisum sativum L.*) (78 000 tonnes), sunflowers (*Helianthus annuus L.*) (73 000 tonnes) and flax (*Linum usitatissimum L.*) (71 000 tonnes) (Statistics Canada, 2015).

These crops are grown in a wide variety of crop sequences. The order of crop sequence can affect crop yield and agronomic outcomes. Wheat following canola (and vice-versa) represent the most popular crop sequence in Manitoba, with 57 percent of spring wheat seeded onto canola stubble, and 51 percent of canola seeded onto spring wheat stubble from 2000 to 2012 (Kubinec, 2012). With the expansion of soybean acres in Manitoba, there are a number of different crops producers are growing before soybean. From 2008 to 2012, the most common crops preceding soybean were spring wheat, oats, canola, soybean, and winter wheat (Table 2.1).

Table 2.1. Frequency of crops preceding soybeans in Manitoba on large acreage fields (>120 acres) from 2008 to 2012 (Yield Manitoba, 2014).

Preceding Crop	Frequency of Sequence (%)
Spring Wheat	22
Canola	21
Soybean	15
Oats	14
Winter Wheat	7
Grain Corn	3
Barley	3
Sunflowers	2

2.2.3 Rotational Yield Benefit

Crop rotations are typically arranged in order to optimize yields. Diversifying a crop rotation can increase soybean yield as well as the yield of other crops in the rotation (Robinson, 1966; Crookston et al., 1988, 1991; Bullock, 1992; Stanger and Lauer, 2008).

A simple crop rotation of two crops has been shown to significantly increase yield over monoculture cropping. In the Mid-Western United States one of the most common rotations is corn-soybean. Rotating soybean with corn has been shown to improve yields of each crop compared to growing either continuously (Crookston et al., 1991). A crop rotation study in Minnesota using conventional tillage by Crookston et al. (1991) compared soybean grown continuously to soybean rotated annually with corn. They found that by the third year of continuous soybean, yield had declined significantly (16-19%) compared to soybean rotated annually with corn (Crookston et al., 1991). The study found that second year continuous soybean yields were equal to yields of soybean rotated annually with corn, indicating that soybean yields may only begin to decline after three or more years of continuous soybean (Crookston et al., 1991). Yield of fourth and fifth year continuous soybean did not differ significantly from third year continuous

soybean, indicating that yield may not continue to decline annually with each successive year of continuous cropping. Soybean grown after several years of continuous corn also yielded higher than soybean rotated annually with corn (Crookston et al., 1991). Meese et al. (1991) found that soybean rotated annually with corn had 15 percent lower yield than soybean grown on two or more years of consecutive corn. This indicates that although there is a rotational benefit from annually rotating between soybean and corn, the rotational benefit to soybean increases the longer a field is not in soybean production.

Increasing rotational diversity beyond a simple two crop rotation can increase yields even more. Davis et al. (2012) compared a two-year corn-soybean rotation to three-year and four-year rotations that also incorporated small grains, red clover (*Trifolium pratense L.*), and alfalfa (*Medicago sativa L.*). They found that soybean yield increased by an average of 9 percent in the three and four-year rotations compared to the two-year rotation. Corn in the three and four-year rotations yielded an average of 4 percent better than the two-year rotation. The three and four-year rotations also reduced fertilizer and herbicide use, and were as economically profitable as the two-year rotation.

The yield impact caused by the rotation effect can last several years. Porter et al. (1997) found that first year soybean following multiple years of corn yielded 18 percent higher than continuous soybean. A second consecutive year of soybean following five years of corn yielded eight percent more than continuous soybean and a third year of soybean yielded three percent more. Fourth and fifth year soybean did not yield more than continuous soybean, indicating the rotational effect had disappeared by this point. Monoculture systems do not achieve the yield benefits attributed to the rotational effect and face multiple challenges that can reduce yield.

In Manitoba, crop sequence has been shown to affect soybean yield. Provincial survey data compiled from large (>120 acres) fields from 2008 to 2012 by Manitoba Agricultural Services Corporation (MASC) shows that soybean grown on soybean yields 95 percent of the provincial average for soybean yield in Manitoba (Table 2.2). Soybean grown on canola, wheat and corn yielded 101, 103 and 107 percent of average soybean yield in Manitoba. This indicates that the previous crop can have a significant impact on soybean yield.

Table 2.2. Influence of previous crop grown on relative soybean yield compared to the provincial average for soybean yield from 2008-2012 (MASC, 2012).

Previous Crop	Relative Soybean Yield (%)
Canola	101
Corn	107
Soybeans	95
Wheat	103

2.2.4 Problems Associated with Continuous Soybean Production

Numerous factors are responsible for poor plant development and yield in continuous soybean cropping rotations. Diseases and pests that occur as a result of continuous soybean production have the greatest impact on yield. Diseases and pests of soybean that are common to the Northern Great Plains that result in significant yield loss include soybean cyst nematode (*Heterodera glycines*), root rot (*Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp.), sclerotinia (*Sclerotinia sclerotiorum*), charcoal root rot (*Macrophomina phaseolina*), sudden death syndrome (*Fusarium verguliforme*), aphids (*Aphis glycines*) and grasshoppers (*Caelifera*) (Wrather et al., 2001; Manitoba Pulse and Soybean Growers, 2015).

Diseases affect yield through root rot, decreased fibrous root branching, decreased nodules and decreased nitrogen fixation (Liu and Herbert, 2002). Several studies have found that continuous soybean cropping increases disease pressure compared to crop rotation by providing a host organism to the pathogen, while rotating non-host crops reduces the incidence of disease (Ross, 1962; Schwartz and Steadman, 1978; Porter et al., 2001; Rousseau et al., 2007). In some cases crop rotation may not affect disease populations when those populations are small or if diseases can persist for several years in the soil. Several studies have found that crop rotation did not reduce sclerotial populations in soybean (Kurle et al., 2001; Mueller et al., 2002). Xu and Yang (1995) found that soybean grown continuously in sterilized soil did not show any decline in development or yield compared to soybean grown in rotation with other crops, suggesting that disease and pests are the most significant source of yield decline in continuous soybean.

A continuous soybean rotation can have a detrimental effect on both soybean development and yield. In terms of growth and development, Liu and Herbert (2002) found that more than two years of continuous soybean cropping in Northern China resulted in reduced plant height, reduced biomass, yellowing of leaves, reduced root development at seedling stage, decreased root nodule numbers, poor canopy development and reduced leaf area index. Soybean residue and roots remaining in the ground after a soybean crop may also inhibit germination and growth of successive soybean seeds (Wang et al., 1995b). Root residuals may also inhibit the growth of seedling roots through inhibition of radicle elongation (Wang et al., 1995a).

The decline in yield has been shown to increase in the third year of continuous soybean, and in some studies has increased even more in the fourth year. Liu and Yu (2000) found that soybean yield in China declined 9.9, 13.8, and 19 percent after two, three and four years of continuous soybean, respectively. Xu et al. (1999) found that soybean yield declined 18.6 percent after two years of continuous soybean and 35.4 percent after three years of continuous soybean, compared to a soybean-corn rotation. The main causes of the decline in yield in these two studies was a decline in pods per plant and seeds per plant caused by an increase in disease incidence (Xu et al., 1999; Liu and Yu, 2000). Crookston et al. (1991), however, found that the decline in soybean yield levelled off after the third year and did not increase thereafter. The majority of studies found a yield loss resulting from continuous soybean from 10-20 percent, although Xu et al. (1999) found that continuous soybean resulted in a yield decline of 35 percent by the third consecutive year of soybeans.

2.2.5 Soil Fertility and Nutrient Depletion

Improved soil fertility is one of the key features of crop rotation. Crops deplete the soil of nutrients in the form of grain harvested and removed. While some nutrients are returned though crop residue, nutrients such as nitrogen and phosphorus can deplete quickly. For example, a 100 bu/ac corn crop in Manitoba is estimated to remove 87-107 lb/ac of nitrogen, while a 35 bu/ac soybean crop removes 130-140 lb/ac of nitrogen (MAFRD, 2007). Although soybeans acquire a significant portion (50-60 percent) of this nitrogen through N_2 fixation, some nitrogen must be replenished to ensure adequate nutrient availability to the subsequent crop. In Europe and North America, this was traditionally done by rotating cereals with nitrogen-fixing legumes, as well as utilizing

livestock manure as a source of nitrogen (Grigg, 1974, 1989). As commercial fertilizers and pesticides gained widespread use in the second half of the twentieth century, some researchers felt that crop rotation would no longer be necessary as fertilizers could replace lost nutrients and pesticides control weeds and insects (Aldrich, 1964; Benson, 1985). Most researchers now believe that crop rotation is an integral management strategy for crop producers (Bullock, 1992). The nitrogen contribution of legumes, combined with the cost of synthetic fertilizers and pesticides is one factor (Bullock, 1992). Reduced disease, weed and insect pressure are also benefits of crop rotation (Bullock, 1992). There are other beneficial effects of rotation that cannot be explained by synthetic fertilizers and pesticides. These benefits are collectively known as the rotation effect (Bullock, 1992).

2.2.5.1 Nitrogen Fixation by Legumes

Legumes fix nitrogen biologically from the atmosphere through a symbiotic relationship with nitrogen-fixing bacteria. Adding legumes to a rotation can reduce the uptake of residual soil nitrogen and in some situations increase available nitrogen to the subsequent crop. Legumes fix and remove nitrogen at varying rates, meaning that some contribute a net nitrogen benefit to the soil after harvest while others remove nitrogen from the soil (Walley et al., 2007). The nitrogen contribution also depends on management; if the crop is harvested for grain the nitrogen contribution will be significantly less compared to a crop that is ploughed down as a green manure. The ability of a plant to fix nitrogen is dependent on: the strength of the symbiotic relationship between host plant and rhizobia, the ability of the plant to acquire soil

nitrogen, soil nitrogen levels, and environmental constraints to nitrogen fixation (Van Kessel and Hartley, 2000).

Although the nitrogen benefit of legumes varies greatly, most legumes fix enough nitrogen that they do not require a nitrogen fertilizer amendment, even if they do not contribute a net nitrogen benefit to the soil (Walley et al., 2007). The high grain protein content of many pulse crops results in a net export of nitrogen, as nitrogen content in the grain often exceeds that fixed biologically (Beck et al., 1991). Various studies have estimated that 45 to 75 percent of nitrogen in above-ground biomass is removed at harvest (Beck et al., 1991; Peoples and Craswell, 1992; Ravuri and Hume, 1993).

Estimates of the nitrogen benefit of different legumes vary. Thiessen Martens et al. (2005) studied the fertilizer replacement value (FRV) of alfalfa relay-cropped with winter cereals, black lentil (*Lens culinaris L.*), red clover and double-cropped chickling vetch (*Lathyrus sativus L.*) to a subsequent oat crop in Manitoba. They found that alfalfa provided the highest FRV (51-62 kg N ha^{-1}), followed by chickling vetch (29-43 kg N ha^{-1}), black lentil (23-39 kg N ha^{-1}), and red clover (24-26 kg N ha^{-1}). Przednowek et al. (2004) compared four different grain legumes in Southern Manitoba (field pea, chickpea (*Cicer arietinum L.*), dry bean and soybean) and found that field pea provided the greatest nitrogen benefit (up to 14 kg N ha^{-1}) to a subsequent wheat crop, while soybean provided almost none.

Mohr et al. (2001) found that timing of termination affected the nitrogen contribution of alfalfa to the following crop in Manitoba. The nitrogen contribution of alfalfa declined the later in the season it was terminated. Termination of the crop before July of the previous year resulted in a nitrogen benefit of 100 kg N ha^{-1}. Termination

from July to August resulted in a nitrogen benefit of 80 kg N ha^{-1}. Termination in fall of the previous year resulted in a benefit of 50 kg N ha^{-1}, while termination in the spring resulted in a benefit of 35 kg N ha^{-1}.

Walley et al. (2007) performed a meta-analysis of nitrogen benefit studies for five pulse crops (common bean, chickpea, lentil, faba bean (*Vicia faba L.*) and pea) on the Northern Great Plains. Nitrogen benefit to the soil was related to N_2 fixation, where crops with a higher percentage of nitrogen derived atmospherically (%Ndfa) contributed more nitrogen to the soil. Generally, nitrogen benefit to the soil was positive when %Ndfa was greater than 48 percent (Walley et al., 2007). Nitrogen benefit is also higher when the harvest index of the crop is lower, although this is usually not desirable (Van Kessel and Hartley, 2000). Faba bean fixed more nitrogen biologically than the other crops and often contributed a net benefit of nitrogen to the soil, while common bean and kabuli chickpea fixed the least nitrogen and usually had a negative nitrogen contribution (Walley et al., 2007). Pea and lentil also had, on average, a positive nitrogen benefit, although less than faba bean (Walley et al., 2007). Campbell et al. (2000) compared a wheat-lentil rotation to continuous wheat over a period of 15 years in southern Saskatchewan and found that the wheat-lentil rotation increased the total soil nitrogen pool at an annual rate of 23 kg ha^{-1}, while continuous wheat only increased the total soil nitrogen pool at a rate of 8 kg ha^{-1}, despite the fact that it received 13 kg ha^{-1} more nitrogen each year.

2.2.5.2 Nitrogen Benefit of Soybean

A number of studies have been performed to determine if soybeans provide a nitrogen benefit to the soil. Przednowek et al. (2004) studied the nitrogen contribution of

soybean to a subsequent wheat crop in Southern Manitoba and found that the nitrogen benefit was very close to zero. A meta-analysis that compiled 637 data sets globally from 1966 to 2006 found that the average net balance of nitrogen in the soil after a soybean crop was -4 kg N ha^{-1} (Salvagiotti et al., 2008). When only the partial nitrogen balance was accounted for (fixed nitrogen in above ground biomass minus nitrogen in seeds), 80 percent of the data sets showed a negative nitrogen balance, averaging a loss of 40 kg N ha^{-1} (Salvagiotti et al., 2008). When the average estimated below ground nitrogen contribution was included, the balance was much closer to neutral. The gap between nitrogen lost and nitrogen produced by fixation increases in high yielding environments (Salvagiotti et al., 2008).

Other studies have found that soybean residue contributes modest amounts of residual nitrogen to the next crop. Toomsan et al. (1995) found that soybean contributed 15 kg N ha^{-1} in total residual nitrogen benefits. Nitrogen benefit to the soil increases as the plant derives more of its nitrogen biologically, but Heichel and Barnes (1984) found that even when 90 percent of nitrogen is fixed biologically, only 24 kg N ha^{-1} is estimated to be returned to the soil.

The N_2 fixation process requires energy in the form of carbohydrates, and therefore it is more efficient for the plant to take up nitrogen available in the soil. Biological nitrogen fixation represents a greater proportion of a crops nitrogen content when soil nitrogen levels are low (Peoples et al., 1995b). It is estimated that 50-60 percent of soybean nitrogen requirements come from the N_2 fixation process, although this number varies from 36 to 74 percent between various studies (Salvagiotti et al., 2008).

Although adding soybean to a rotation may not always constitute a nitrogen benefit, soybeans can reduce the need for nitrogen inputs for subsequent crops. Nitrogen amendments are not normally required for soybeans as they biologically fix 50-60 percent of their nitrogen requirement (Salvagiotti et al., 2008). Several studies have shown little to no increase in soybean yield by applying nitrogen fertilizer to a soybean crop (Welch et al., 1973; Freeborn et al., 2001; Barker and Sawyer, 2005). Adding soybean to a rotation can also decrease nitrogen requirements for the following year's crop. Growing soybean before corn can decrease the amount of nitrogen required for the following corn crop by 40 pounds per acre compared to growing corn before corn (Roth, 1996). Soybean, wheat and canola all have lower carbon to nitrogen (C:N) ratios than corn (MAFRD, 2007; Gan et al., 2011) Although most studies show soybean does not contribute nitrogen to the soil, its lower C:N ratio compared to corn results in less nitrogen immobilized by soil microbes and rendered unavailable to the subsequent crop (Green and Blackmer, 1994).

2.2.6 Soybean Residue

Residue management is an important component of crop rotation. Residue can affect nutrient immobilization, seed placement, soil erosion, disease levels, soil temperature and moisture for subsequent crops (Roth, 1996). Residue breakdown and subsequent nutrient immobilization and mineralization is affected by the C:N ratio of the crop. Carbon to nitrogen ratios of crops vary (Table 2.3).

Carbon to nitrogen ratios affect the soil microbial community and the immobilization and mineralization of carbon and nitrogen. Microbial organisms take up carbon and nitrogen at a rate of approximately 25:1 (United States Department of

Agriculture, 2011). Crop residues with a C:N ratio lower than 25:1 have enough nitrogen to supply soil microbes. Residues that have a higher C:N ratio do not have enough nitrogen relative to carbon and therefore microbes begin to immobilize soil nitrogen, rendering it unavailable to plants (United States Department of Agriculture, 2011).

Although immobilization is not a permanent loss of nitrogen, it is unavailable until the microbes die and decompose. Gentry et al. (2001) found that net soil nitrogen mineralization in corn following corn was 33 kg ha^{-1} lower than in corn following soybean, indicating that greater immobilization of soil nitrogen had occurred in corn. This results in lower nitrogen requirements for corn following soybean compared to continuous corn (Nafziger et al., 1984; Peterson and Varvel, 1988).

Table 2.3. Average crop residue Carbon:Nitrogen (C:N) ratios and residue amounts in Manitoba.

Crop Residue	C:N Ratio (Range)	Residue Amounts (kg ha^{-1})
Corn Stover	82:1 (65-95:1)[1]	7400
Soybean Residue	65:1[1]	3700
Canola Straw	33:1[2]	N/A
Wheat Straw	60:1 (35-85:1)[1]	2500-3700

[1] Estimates from the Manitoba Soil Fertility Guide (2007)
[2] Estimate from Agriculture and Agri-Food Canada (2011)

On average, above-ground soybean residue in Manitoba is 3700 kg ha^{-1} (Heard, 2006). This is significantly less than corn, which produces 7400 kg ha^{-1}. This large amount of corn residue can accumulate, disrupting proper seed placement, increasing the potential for disease, decreasing soil temperature and increasing soil moisture (Roth, 1996). This can be particularly problematic in no-till cropping systems as residue is not

readily broken down. Crops that produce less residue may be easier to manage in no-till systems.

The relatively small amount of soybean residue does not protect the soil from erosion as well as other crops that produce more residue. Cogo et al. (1983) found that corn residue was more effective at preventing erosion from rainfall than soybean residue. Dickey et al. (1985) compared total soil loss in soybean residue and corn residue after a rainfall event. They found that soybean residue had 40 percent greater total soil loss than corn residue on a silty clay loam soil with a 5 percent slope. Total soil loss was even greater on a silt loam soil with a 10 percent slope, ranging from 50 to 1100 percent greater in soybean residue compared to corn. They found that no-till soybean reduced soil loss from erosion by 50 percent compared to a cleanly tilled soil, indicating that soybean residue still provides some degree of protection from erosion.

2.2.6.1 The Rotation Effect

Although nitrogen contribution of legumes is an important component of crop rotation, it does not completely explain the yield benefit seen as a result of rotation. An important component of the rotation effect is soil organic matter (SOM). Continuous cropping tends to result in lower levels of SOM compared to a rotation of crops. Rotations that incorporate sod, pasture or hay crops increase SOM relative to rotations that do not (Bullock, 1992). Although short rotations such as corn-soybean still provide a rotation effect, they decrease SOM at a faster rate than rotations that incorporate pasture or hay (Dick et al., 1986). Dick et al. (1986) found that a corn-oat-alfalfa rotation had higher SOM than a corn-soybean rotation, while a continuous soybean rotation had even lower SOM levels. Havlin et al. (1990) also found that a continuous soybean rotation had

lower organic carbon and nitrogen compared to a sorghum-soybean rotation or continuous soybean. Soybean in particular can reduce SOM as it does not produce as much biomass as corn (Dick et al., 1986). In Manitoba, corn residue dry matter is 7400 kg ha^{-1}, while soybean residue dry matter is only 3700 kg ha^{-1} (MAFRD, 2007). High residue producing crops such as wheat and sorghum in rotation with soybean have been found to increase soil carbon and nitrogen (Kelley et al., 2003).

Soil organic matter improves soil quality through improved mineral availability (Allison, 1973; MacRae and Mehuys, 1985), bulk density (DeKimpe et al., 1982), aggregate formation and stabilization (Fahad et al., 1982; MacRae and Mehuys, 1985), water retention capacity (Jamison, 1953), water infiltration rate (Wischmeier and Mannering, 1965; Allison, 1973), soil aeration and decreased soil erosion (Bezdicek, 1984). These improvements in soil structure and quality are an important component of the rotation effect. This applies to soybean in rotation. Zuber et al. (2015) found that a corn-soybean-wheat rotation in Illinois had higher water aggregate stability, total nitrogen and potassium levels compared to corn-soybean-corn and continuous soybean.

Soil microorganisms are also an important component of the rotation effect. Crop rotation has been shown to increase soil enzymatic activity (a measure of microbial activity) compared to continuous cropping (Kahn, 1970; Gauger, 1987). Increases in soil enzyme activity are closely related to changes in SOM (Gantzer et al., 1987). Extending the length and diversity of crop rotation can reduce the presence of deleterious rhizosphere microorganisms and increase the presence of beneficial ones (Schippers et al., 1987).

2.2.7 Phosphate Depletion by Soybean

Crop production removes soil phosphorus in the grain harvested. Soil phosphorus levels have been declining in many parts of Manitoba. Soil phosphorus levels tested below critical levels (less than 20 ppm) in 64 percent of soil samples taken in Manitoba (International Plant Nutrition Institute, 2015). Soybean is a significant consumer of phosphorus and applying large amounts of phosphorus fertilizer increases the risk of fertilizer toxicity to the soybean plant (Hanway and Weber, 1971; Heard, 2006; Bardella, 2016). The safe rate of seed-placed phosphate for soybean is 11 kg ha^{-1} (MAFRD, 2007). Recent research from Manitoba shows a soybean crop of 45 kg ha^{-1} will remove 38 kg P_20_5 ha^{-1} (Bardella, 2016). Research from Manitoba shows that 85 percent of phosphorus uptake by soybean is removed in the seed (Heard, 2006). Soybeans are efficient users of phosphorus and do not show a strong response to phosphorus fertilization, as they are able to take up soil phosphorus even when levels are low. This indicates that the depletion of phosphorus by the soybean crop is more likely to affect future crops than soybean.

2.2.8 Diseases and Pests

Soybeans are prone to a variety of diseases and pests. Common soybean diseases in Manitoba include seedling diseases (*Phytophthora spp.*, *Pythium spp.*, *Rhizoctonia spp.* and *Fusarium spp.*), sclerotinia (*Sclerotinia sclerotiorum*), powdery mildew (*Microsphaera diffusa*), bacterial blight (*Pseudomonas syringae pv. glycinea*), downy mildew (*Peronospora manshurica*), septoria brownspot (*Septoria glycines*), phyllosticta leaf spot (*Phyllosticta sojicola*), and pod and stem blights (*Diaporthe phaseolorum*) (Manitoba Pulse and Soybean Growers, 2015). Common insects include grasshoppers,

soybean aphids, seedcorn maggots (*Delia platura*), cutworms (*Agrotis spp.*), wireworms (*Elateridae spp.*), two-spotted spider mites (*Tetranychus urticae*), green cloverworm (*Hypena scabra*) and corn earworm (*Helicoverpa zea*) (Manitoba Pulse and Soybean Growers, 2015).

Crop rotation is an effective method of controlling diseases and pests. Monoculture cropping allows pathogen populations to persist and build up in the soil. Disease populations decline when crops are grown that do not act as a host to the pathogen (Bullock, 1992). Some pathogens can have multiple host crops, and certain crop rotations control pests more effectively than others. Soybean root rot diseases such as *Phytophtora* and *Pythium* can be controlled by rotating soybean with corn and wheat, where their populations do not thrive (Anonymous, 2009). A longer rotation will result in lower disease populations and fewer races present (Bullock, 1992).

2.2.9 Crop Rotation Summary

Crop rotation has been shown to have many beneficial effects on plant growth and development, yield, soil fertility, disease incidence and residue management. Rotation also plays an important role in how soil biota function. Rotation can have an important effect on the presence and development of arbuscular mycorrhizal fungi and *Bradyrhizobium*.

2.3 Arbuscular Mycorrhizal Fungi

2.3.1 Introduction

Arbuscular mycorrhizal fungi (AMF) are a fungus of the order *Glomales* that infect the root system of many vascular plants, forming a mutually symbiotic relationship wherein soil nutrients are transferred to the plant in exchange for plant carbohydrates (as reviewed by Smith and Read, 2008). Infection of roots by AMF is one of the oldest symbiotic processes known, dating back over 400 million years to the early Devonian age (Remy et al., 1994). An estimated 80 percent of extant vascular plants on earth form some type of mycorrhizal symbiosis and can benefit from this relationship (Schüβler et al., 2001). A number of plants are non-mycorrhizal and do not readily form associations with mycorrhizae. As such, crop rotation can impact AMF colonization negatively when non-mycorrhizal crops are grown. In Manitoba, the most commonly grown non-mycorrhizal crop is canola (McGonigle, 2009).

AMF are obligate symbionts and cannot survive without colonizing a host (as reviewed by Smith and Read, 2008). The most important nutrient acquisition that AMF provide to plants is the transfer of phosphorus, but AMF can also be an important source of water, nitrogen, copper, potassium and zinc (Marschner and Dell, 1994). AMF can also provide a number of benefits to an agroecosystem, including improved soil aggregate stability (Bearden and Petersen, 2000; Miller and Yastrow, 2000), protection against detrimental pathogens such as fungi and nematodes (Elsen et al., 2001; Veresoglou and Rillig, 2012), and inhibition of growth of non-mycorrhizal weeds (Jordan et al., 2000).

2.3.2 Physiology

Arbuscular mycorrhizal fungi are named after their arbuscules and vesicles, two prominent structures of the organism that are unique to the *Glomeromycota* phylum (as reviewed by Smith and Read, 2008). The other physical structures of AMF include hyphae and spores.

2.3.2.1 Hyphae

Hyphae are long, branching filamentous structures that perform a variety of functions including nutrient acquisition, carbohydrate breakdown and root infection. They exist as both extraradicle hyphae (outside the root cortex) or intraradicle hyphae (inside the root cortex) (as reviewed by Smith and Read, 2008). Extraradicle hyphae initiate infection of the host root and play a crucial role in delivering phosphorus to the plant. There are two types of extraradicle hyphae. Absorptive hyphae are thinly branched hyphae that are responsible for nutrient uptake from the soil. Distributive hyphae extend the influence of the hyphal network into the soil and act as nutrient conduits (as reviewed by Smith and Read, 2008). Hyphae are one to two orders of magnitude smaller in diameter than roots, and as such have the ability to access soil pores and nutrients that are unavailable to roots (as reviewed by Smith and Read, 2008). External hyphae vastly increase the absorbing surface area of a root system, increasing contact with soil and nutrients and allowing for increased nutrient uptake (Marschner and Dell, 1994). Although the diameter of most extraradicle hyphae are only 2-10 μm, compared to 300 μm and greater for roots, the radius of influence of the mycelial network can be up to 250 cm from the root, vastly increasing access to soil volume (as reviewed by Smith and Read, 2008).

2.3.2.2 Arbuscules

Arbuscules are very finely branched haustoria that act as the major site of nutrient exchange between the plant and AMF (as reviewed by Smith and Read, 2008). They exist inside the cells of the root cortex (although remain outside the cytoplasm), developing from intercellular hyphae (as reviewed Smith and Read, 2008). Arbuscules form as early as two days after the initial infection. They are short lived, surviving for only a few days before degrading. Growth begins with a trunk branch that is 5-10 µm in diameter. As arbuscules continue to grow the branches become increasingly finer, reaching less than 1 µm in diameter. They are the location of the vast majority of nutrient transfer to the plant and carbon transfer to the fungi (as reviewed by Smith and Read, 2008).

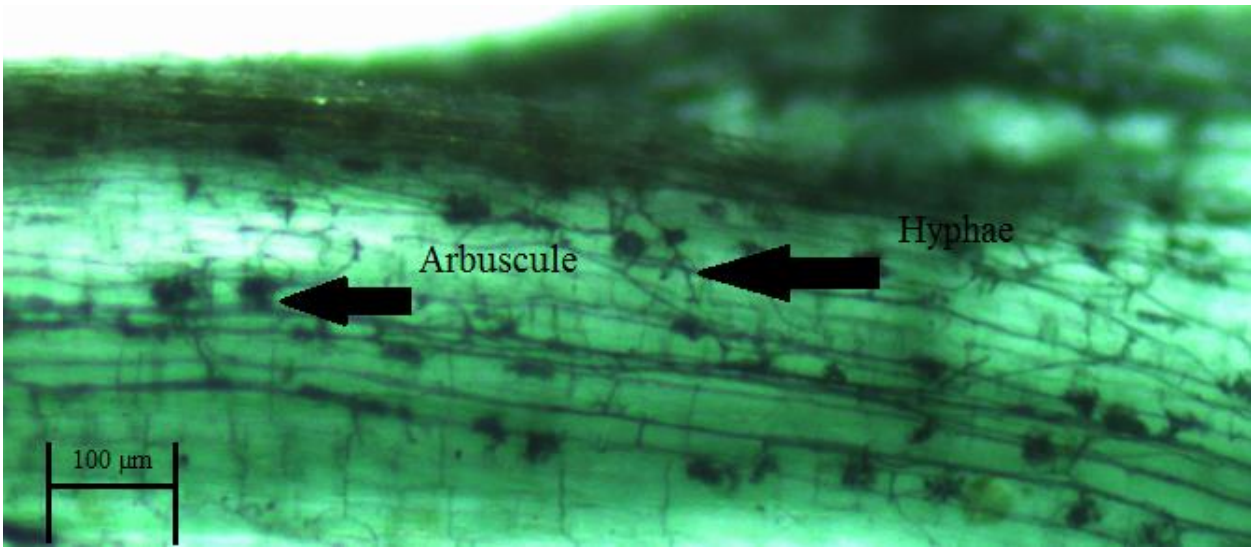

Figure 2.1. Hyphae and arbuscules of AMF in soybean root (Sanders, 2016).

2.3.2.3 Vesicles

Vesicles are storage organs in AMF. They accumulate large amounts of lipids and cytoplasm that can be used as an energy source as necessary. They form as swellings on the hyphae soon after the first arbuscules develop either intra or intercellularly. They have been known to act as propagules and to assist in regrowth of intercellular hyphae (as reviewed by Smith and Read, 2008). While the majority (80 percent) of AMF species form vesicles, there are some AMF that do not (as reviewed by Smith and Read, 2008).

2.3.2.4 Spores

Spores function as both a storage structure and as one of the main propagules of AMF. They form as a swelling in the hyphae containing lipids, cytoplasm and haploid nuclei (as reviewed by Smith and Read, 2008). They are very large relative to other organs of AMF (up to 500 μm in diameter). They have thick walls made of chitin and sometimes glucan and can persist in soil for relatively long periods of time. Inside the spores are a large number of nuclei, ranging from 800 to 35 000 depending on species (Hosny et al., 1998). Spores do not require the presence of root exudates from host plants to germinate, although they do appear to stimulate germination (Graham et al., 1982). When spores germinate, hyphae extend from the spore wall in search of a root to colonize, using spore reserves as its energy source (as reviewed by Smith and Read, 2008). If a host is not found, and in the absence of host signalling molecules, hyphal growth will cease to conserve energy (Giovannetti et al., 1993). Although they often function as propagules spore density is not necessarily indicative of the extent of root colonization (as reviewed by Smith and Read, 2008).

2.3.3 Symbiotic Relationship of Mycorrhizae with Plants

AMF are an obligately symbiotic organism. They cannot survive for long periods without an autotrophic host plant, and the vast majority of attempts to produce viable spores without a symbiotic host have failed (as reviewed by Smith and Read, 2008). In general, plants that have coarser, less fibrous root systems (such as species with tap roots) tend to depend more on mycorrhizae as their roots cannot access as much surface area as thinner, more fibrous roots (Johnson et al., 1997). Plants with a higher shoot to root ratio also tend to be more dependent on AMF as they have fewer roots taking up less surface area to find soil nutrients (Johnson et al., 1997). The symbiotic relationship begins with host recognition by AMF and continues through the infection process, hyphal and arbuscular growth, and nutrient exchange.

2.3.3.1 Mechanisms of Infection

Communication between host plant and AMF is critical for successful symbiosis. Plants release root exudates that signal the presence of a host to AMF hyphae in the soil (as reviewed by Smith and Read, 2008). One of the most important signalling molecules is strigolactone, a carotenoid derived hormone that stimulates branching and growth of the hyphae. Low phosphorus in the soil stimulates strigolactone release from the plant (as reviewed by Smith and Read, 2008). When strigolactone comes into contact with AMF, it stimulates hyphal branching by inducing the transcription of genes involved in respiratory pathways and mitochondrial activity (as reviewed by Smith and Read, 2008). Only small concentrations of strigolactone are required to stimulate branching. Hyphae continue to branch out until a host root is encountered. As the hyphae approaches the

root it branches out profusely in order to form more infection sites (as reviewed by Smith and Read, 2008).

When extraradicle hyphae come into contact with a host root they adhere to the root and grow along its surface. After several days a structure known as an appressorium forms on the surface of the root cortex (as reviewed by Smith and Read, 2008). The plant responds to this swelling with several morphological changes. Epidermal cells in the root separate to allow hyphae to penetrate. The hyphae itself narrows at the tip to form an infection peg, facilitating penetration. Once penetration has occurred the tip of the hyphae will expand again. Mycorrhizae are believed to release pectinase enzymes at this time to break down root cell walls and facilitate penetration. Short cells in the exodermis of the root have less suberin, allowing for hyphae to penetrate through the root more easily. In ideal conditions, root penetration occurs about two days after the initial contact. Once penetration has occurred hyphae branch out into the middle and inner cortex of the root (as reviewed by Smith and Read, 2008). Within two days arbuscules begin to form and the infection process has been successfully completed.

2.3.3.2 Nutrient Uptake

Mycorrhizae are capable of providing the host plant with a variety of nutrients, often in significant quantities. Although the proportion of plant nutrients derived from AMF symbiosis depends on a variety of external and internal factors, AMF are capable of providing up to 80 percent of plant phosphorus, 25 percent of plant nitrogen, 10 percent of plant potassium, 60 percent of plant copper, and 25 percent of plant zinc (Marschner and Dell, 1994). The level of uptake varies greatly based on plant species, AMF species and soil nutrient concentration. Mycorrhizae can also provide a host plant with low

amounts of sulfur, calcium and iron, although not in significant quantities. (Marschner and Dell, 1994).

2.3.3.2.1 Phosphorus Uptake

Phosphorus uptake is the most important component of mycorrhizal symbiosis. Phosphorus is required in plants in fairly large quantities, but is often present in the soil at limiting amounts and is poorly labile. Phosphorus can be immobilized by bacteria or fixed as iron, aluminum or calcium phosphate, rendering it unavailable to plants (as reviewed by Smith and Read, 2008). Although these forms of phosphorus can eventually be made available to the plant, this is a very slow process that can take many years. Phosphorus has a patchy distribution throughout the soil profile, which can reduce availability to the plant. Inorganic phosphate is negatively charged and the inside-negative electrical potential of cell membranes requires a large active energy contribution from plant roots or fungi in order to actively take up phosphorus (Smith and Smith, 2012). Plant roots take up phosphorus that is immediately available, but this creates a depletion zone in the rhizosphere surrounding the root (as reviewed by Smith and Read, 2008). The hyphal network extends beyond these depletion zones, allowing the plant to expand its rhizosphere and access zones that are comparatively rich in phosphorus.

Uptake of phosphorus by AMF is more efficient than uptake by roots. The production of hyphae requires a smaller expenditure of carbon per unit of length than a plant root (Tinker, 1975). The excretion of hydrogen ions by hyphae lowers soil pH, increasing the solubility of phosphorus and decreasing the chelating ability of organic anions (Smith, 1980). Research using isotopically labelled phosphorus markers has shown that phosphorus uptake by AMF is the major route of uptake in most plants, even

when there is not an increase in phosphorus content compared to a control (as reviewed by Smith and Read, 2008). AMF have the ability to reduce direct phosphorus absorption by plants roots through downregulation of phosphorus transporter hormones in the root (as reviewed by Smith and Read, 2008).

Orthophosphate is actively taken up into the extraradicle mycelium in the form of $H_2PO_4^-$ with the help of phosphate transporter genes (as reviewed by Smith and Read, 2008). Phosphorus is then stored in vacuoles in the form of polyphosphate. The actual transfer of phosphorus from AMF to plant involves the use of membrane transport steps located at interfaces between the two organisms (as reviewed by Smith and Read, 2008). Mature arbuscules are the location of these interfaces and this is where the majority of transfer occurs.

Mycorrhizae also provide plants with a variety of other nutrients, although there is considerably less known about AMF's role in the uptake of other nutrients as this is considered of secondary benefit to its role in plant phosphorus acquisition. Mycorrhizae tend to provide the most benefit in helping with uptake of nutrients that are less mobile in the soil (Johnson et al., 1997).

2.3.3.2.2 Nitrogen Uptake

Mycorrhizae can provide a significant portion of plant nitrogen. Using compartmented vessels and ^{15}N to measure hyphal nitrogen uptake, Ames et al. (1983) found that celery (*Apium graveolens L.*) inoculated with AMF derived 25 percent of its nitrogen from ^{15}N accessed by hyphae in the compartmentalized vessel, while non-mycorrhizal celery derived just 3.5 percent of its nitrogen from the same source. There were no differences between mycorrhizal celery and non-mycorrhizal celery in terms of

total nitrogen or shoot dry weight, indicating that roots responded to increased hyphal nitrogen uptake by decreasing their own uptake. A similar experiment involving couch grass (*Elymus repens L.*) found that 24 percent of the plant's nitrogen was derived from external hyphae (George et al., 1992).

Nitrogen is taken up by external hyphae in the form of NH_4^+ and NO_3^-. The uptake of NH_4^+ contributes to the acidification of the soil which in turn renders phosphorus more soluble and available for uptake (as reviewed by Smith and Read, 2008). While NH_4^+ and NO_3^- accumulate in extraradicle mycelium in the form of amino acids such as arginine, when nitrogen is transferred from AMF to the plant the arginine compound is broken down and nitrogen is transferred to the plant in the form of inorganic ammonium, while the other components of arginine are recycled back into the fungal network (as reviewed by Smith and Read, 2008).

2.3.3.2.3 Carbon Transfer

Mycorrhizae are entirely dependent on their host for energy, which they derive from the plant in the form of carbohydrates (as reviewed by Smith and Read, 2008). It has been estimated that the percent of a plant's carbon supply allocated to AMF can range from 4-20 percent (Peng et al., 1993). Plants provide carbon to AMF in the form of hexoses that can be broken down into easily digestible sugars that are used as a quick and readily available source of energy (as reviewed by Smith and Read, 2008). Carbon exchange can occur either inter or intracellularly and between hyphae or arbuscules, but arbuscules are considered the main point of carbon exchange. The interfaces where exchange occurs utilize a plasma membrane separated by an apoplastic interfacial compartment (as reviewed by Smith and Read, 2008). The nutrient exchange process is

not passive, it requires active efflux and uptake from both partners as there is no cytoplasmic continuity between the two symbionts. It is believed that nutrient exchange to the plant and carbon exchange to AMF occur along separate interfaces and operate along the same chemical potential gradients that govern other plant processes (as reviewed by Smith and Read, 2008).

Once hexoses from the host are in AMF, they are rapidly broken down into trehalose and glycogen. This prevents excess glucose accumulation in the cytoplasm (Shachar-hill et al., 1995). Lipid synthesis also occurs in the intraradicle hyphae. These products are then sent to extraradicle hyphae to further growth. This process is especially important to sustain growth early during the infection process. Before the symbiosis is fully formed AMF can absorb small amounts of hexose or acetate. As the symbiosis develops AMF are able to absorb hexoses more rapidly, with glucose being more readily absorbed than fructose (as reviewed by Smith and Read, 2008). Hyphal networks can form complex webs between plants wherein carbon can be transferred from a donor plant to a recipient plant (Francis and Read, 1984). This exchange allows plants to exchange organic carbon amongst themselves as required.

2.3.4 Factors Affecting Mycorrhizal Colonization

2.3.4.1 Crop Rotation

Crop rotation plays a significant role in mycorrhizal colonization. Although most terrestrial plant species are mycorrhizal, several major families are not. These include members of the *Brassicaceae*, *Chenopodiaceae*, *Caryophyllaceae*, *Polygonaceae*, *Juncaceae* and *Proteaceae* families (as reviewed by Smith and Read, 2008). In Manitoba, canola (*Brassica napus*) is the most common crop that does not form a

symbiotic relationship with AMF. Common weeds in Manitoba that are non-mycorrhizal or weakly mycorrhizal include volunteer canola, wild mustard (*Sinapis arvensis L.*), lamb's quarters (*Chenopodium album L.*), smartweed (*Polygonum spp.*) and buckwheat (*Fagopyrum esculentum L.*) (as reviewed by Smith and Read, 2008).

Numerous studies have shown that including a non-mycorrhizal species in a crop rotation will reduce AMF colonization in subsequent crops (Tommerup, 1984; Harinikumar and Bagyaraj, 1988; Douds et al., 1997; Gavito and Miller, 1998; Karasawa et al., 2002; McGonigle, 2009; Chen et al., 2013). Mycorrhizal colonization can be reduced significantly after just one year growing a non-mycorrhizal crop. Chen et al. (2013) found that soybean following canola or sugar beet (*Beta vulgaris L.*) (both non-mycorrhizal) had significantly lower percent root colonization (14-18 percent) than soybean grown on corn, wheat, Illinois bundleflower (*Desmanthus illinoensis L.*), soybean, alfalfa, sunn hemp (*Crotalaria juncaea L.*) or sunflower. McGonigle (2009) found that flax grown in Manitoba had higher AMF colonization and phosphorus concentration following wheat than canola. A study conducted in Ontario found that growing canola before corn significantly reduced AMF colonization in corn compared to bromegrass (*Bromus spp.*), alfalfa or corn (Gavito and Miller, 1998). The effect of crop rotation on AMF colonization was significantly greater than tillage amendments or phosphorus application, both of which are known to affect colonization. The results remained the same in both a field trial and a bioassay. Cropping a non-mycorrhizal and poorly mycorrhizal crop (*Spinaceae oleraceae* and *Capsicum annum*, respectively) was found to significantly reduce both mycorrhizal colonization and spore populations in corn plants compared to a mycorrhizal crop (Douds et al., 1997). Conversely, corn grown

after soybeans did not have less AMF colonization than continuous corn (Tian et al., 2013).

Although growing a non-mycorrhizal crop results in a decrease in AMF colonization, leaving land fallow can result in a greater decrease in colonization. Harinikumar and Bagyaraj (1988) found that growing non-mycorrhizal mustard before cowpea (*Vigna unguiculata L.*) reduced mycorrhizal propagules by 13 percent. Leaving land fallow reduced mycorrhizal propagules by 40 percent (Harinikumar and Bagyaraj, 1988). Black and Tinker (1979) found that one year of fallow or one year of non-mycorrhizal kale (*Brassica oleraceae L.*) both reduced colonization in barley by 50 percent.

2.3.4.2 Soil Characteristics

Mycorrhizae are affected by a wide range of soil characteristics and respond differently to specific soil conditions. Some of these factors include: phosphorus fertility, soil type, aeration and soil temperature. Mycorrhizae thrive in nutrient deficient soils where plants become dependent on the symbiosis for nutrient uptake (Siqueira and Saggin-Junior, 1995). Several studies have shown that AMF colonization is higher in low fertility soils, especially in relation to phosphorus. Clay soils tend to adsorb ions from the soil more efficiently, making them unavailable for plant uptake, and as such tend to be more fertile than sandy soils. Carrenho et al. (2007) found that AMF colonization of sorghum was significantly lower in a clay soil than a sandy soil. Phosphorus is more readily available in sandy soils as it is not adsorbed as much as in clay soils. This can result in a decline in colonization.

Clay soils encourage the formation of suberin, a waxy substance that prevents water from entering root tissue. This makes root penetration by the hyphae more difficult, potentially inhibiting colonization (Koske and Gemma, 1995). Good soil aeration allows hyphae to move more freely through the rhizosphere and encourages colonization (Saif, 1983). Optimal soil temperatures (30-35°C) favor spore germination and hyphal growth (Tommerup, 1983). As sandy soils are generally warmer than clay soils this tends to promote AMF colonization over clay soils.

2.3.4.3 Phosphorus Fertility and Application

Numerous studies have found that phosphorus fertility and fertilizer application has a detrimental effect on mycorrhizal colonization. Treseder (2004) compiled global data from 20 long term phosphorus fertilization studies and found that phosphorus fertilization consistently reduced mycorrhizal colonization. There was an average 32 percent decline in mycorrhizal colonization following phosphorus application across the studies. The rate of phosphorus application is significant in determining the effect it will have on AMF colonization, and subsequently root, shoot and total biomass. Oliver et al. (1983) found that AMF colonized subterranean clover (*Trifolium subterraneum L.*) plants produced greater root, shoot and total dry weight biomass compared to non-colonized plants at phosphorus application rates of 0 and 6.2 mg P/kg, but at rates of 12.4 and 20.8 mg P/kg the non-colonized plants produced greater dry weight biomass. This indicates that at higher levels of phosphorus application the symbiotic relationship becomes a net drain on resources to the plant, and becomes parasitic.

Phosphorus fertilization has been found to have a significant effect on mycorrhizal colonization even at low levels of application. Clapperton et al. (1997)

found that phosphorus applied at 20 kg ha^{-1} yr^{-1} over twenty years significantly reduced uptake of copper, zinc and calcium by wheat. Application of phosphorus also resulted in significantly less root colonization by AMF and reduced length of root colonization. Tang et al. (2001) found that cattail (*Typha angustifolia L.*) grown with phosphorus concentration treatments of 1, 10, 100 and 500 µM resulted in some degree of mycorrhizal colonization at the lowest three phosphorus concentrations but not at the highest concentration of phosphorus. Colonization rates did not differ significantly between spore inoculum densities of 200 or 500 spores per pot, indicating that phosphorus application has a stronger effect on colonization rate than spore density. Proportional root colonization was significantly higher in the 1 and 10 µM treatment compared to the higher phosphorus concentrations, but root and shoot biomass was significantly greater in the 100 µM treatment than lower phosphorus concentrations. Although phosphorus rates did affect mycorrhizal colonization, increased colonization did not necessarily result in greater root and shoot biomass. Some studies have suggested that the increased phosphorus availability in wetter conditions results in decreased mycorrhizal colonization (Rubio et al., 1997), although whether colonization was inhibited as a result of phosphorus availability or excess moisture remains unclear. The effect of timing of phosphorus application is also poorly understood. Further research is required to determine if the timing of application plays a role in the degree to which mycorrhizae can effectively colonize roots.

2.3.4.4 Nitrogen Application

Although not as significant as phosphorus application, nitrogen application can also affect mycorrhizal colonization. Treseder (2004) published a comprehensive

analysis of 31 field studies of nitrogen fertilization and its effect on mycorrhizal colonization. The field studies were conducted on a range of biomes including agricultural, temperate grassland, woodland, temperate forest, boreal forest and tropical forest. The meta-analysis found that across studies nitrogen fertilization resulted in an average 15 percent decline in mycorrhizal colonization. This is compared to a 32 percent average decline for phosphorus application. The effect of application rate was only significant at extremely high application rates of 1000 kg N ha^{-1} yr^{-1}. Nitrogen is very rarely applied at such a high rate in agricultural crops and it is unlikely that nitrogen application will have an effect on AMF colonization in most conventional cropping systems.

Fertilization type, mycorrhizal species, and duration of fertilization had no significant effects on colonization. The nitrogen fertilization studies were much less consistent in their results than the phosphorus fertilization studies. Twenty-three percent of nitrogen studies found that applying nitrogen actually increased colonization. Treseder and Allen (2002) found that nitrogen application increased AMF biomass at nitrogen-limiting sites, suggesting that there is a threshold for soil nitrogen wherein AMF become limited. Mycorrhizae depend on nitrogen and phosphorus for growth just as plants do and therefore some nitrogen application in a nutrient deficient site may actually be beneficial.

2.3.4.5 Tillage

Tillage has also been shown to decrease AMF colonization. The mechanical damage from tillage disrupts the hyphal network in the soil, inhibiting colonization (Abbott and Robson, 1991). Tillage can also increase erosion, which decreases

mycorrhizal propagules and limits colonization. Tillage using more intensive implements has been shown to significantly reduced AMF colonization compared to less intrusive tillage methods (Vivekandanan and Fixen, 1991).

2.3.5 Effect on Yield

The effect of AMF on plant biomass and crop yield varies greatly depending on the degree to which the host plant is mycotrophic. Some crops (such as flax) are heavily dependent upon AMF for phosphorus and growth, while others (such as canola) show very little propensity to form a symbiotic relationship with AMF (Gerdemann, 1968). The soil environment is also a critical determining factor in the role AMF will play. AMF have the greatest positive impact on yield in environments where nutrients such as phosphorus are limited (Lekberg and Koide, 2005). Adequate phosphorus is particularly critical early in the growing season and plants that do not receive enough phosphorus at this stage suffer reduced crop yield (Grant et al., 2001).

Several meta-analyses have shown that AMF generally increase plant growth and yield. McGonigle (1988) conducted a meta-analysis consisting of 78 field trials from 27 experiments studying a variety of crops from around the world. He concluded that an increase in AMF colonization (via inoculation of those crops) resulted in an average yield increase of 37 percent. Of the 78 field trials analyzed, fifteen trials showed a decline in yield with increased AMF colonization, while five trials had no effect. Lekberg and Koide (2005) conducted a meta-analysis of 290 field and greenhouse trials taken from 71 studies conducted on various crops around the world. Increases in AMF colonization were accomplished through inoculation, shortened fallow, or reduced soil disturbance. They found that an increase in AMF colonization resulted in an average yield increase of

23 percent. The method through which AMF colonization was increased significantly affected yield results. Studies where the method of increasing AMF colonization was inoculation had an average yield increase of 34 percent. Studies where the method was shortened fallow had an average yield increase of 27 percent. Studies where the method was reduced soil disturbance had an average yield reduction of 3 percent.

Mycorrhizae have been shown to increase soybean yield. Young et al. (1986) found that inoculation of soil with AMF increased soybean yield by 7-45 percent depending on soil type. Phosphorus content of the grain was also significantly increased. The degree of yield increase varied by mycorrhizal strain, with *G. fasciculatum* resulting in the greatest increase in yield. Leaves of non-inoculated plants began to senesce sooner, which could potentially reduce photosynthate supply to the pods and seeds, reducing yield. This study was conducted using pots in a greenhouse, and the effect of mycorrhizal colonization on yield in a complex soil ecosystem may be less clear.

In a greenhouse study, Bethlenfalvay et al. (1997) compared soybean that had been inoculated with AMF to plants that were not inoculated but received a phosphorus fertilizer amendment and a control that received no phosphorus amendment and no inoculant. They found that seed yield was 65 percent greater in plants that were inoculated compared to plants that were not inoculated and had not received a phosphorus amendment. Plants that were not inoculated and did receive a phosphorus amendment, however, yielded 42 percent higher than the plants that were inoculated with no phosphorus amendment. This indicates the importance of phosphorus in determining the effect of AMF on soybean seed yield.

2.3.6 Assessment of Arbuscular Mycorrhizal Fungi

In order to estimate the level of nutrient exchange occurring between a host and its AMF symbiont, researchers must first estimate the quantity of AMF present in the root. There are a variety of methods for assessing the presence of AMF dating back to the 1970s (Treseder, 2013). Most estimation methods involve some degree of visual quantification by the researcher. Hyphae, arbuscules and vesicles can be distinguished by their defining characteristics under a microscope. The value inherent in this system is that it allows for identification of key features such as arbuscules. There are several limitations to visual systems of quantification. Visual quantification can sometimes be difficult because cortical cells and parts of the stele can become stained, making it difficult to distinguish AMF from these parts of the root (Dodd and Jeffries, 1986). Smaller arbuscules can be difficult to detect and arbuscules can be confused with structures formed by other fungi (McGonigle et al., 1990). Thus, there is also a degree of subjectivity involved in visual quantification of AMF.

2.3.6.1 Gridline Intersect Method

The gridline intersect method is the most common method of visual quantification for assessing AMF colonization (McGonigle et al., 1990). Roots are cleared using an autoclave or similar device, rendering them more transparent. Larger structural roots are usually removed as they do not clear well (Giovannetti and Mosse, 1980). Roots are then soaked in KOH to soften them, which allows a staining agent such as Trypan blue or Chlorazol black E to bind to fungal structures, making them visible under a microscope (Vierheilig et al., 2005). The stained roots are then placed on a Petri dish with a gridline marked on the bottom of the dish. Roots are viewed at 40X to 100X magnification.

Horizontal gridlines are scanned and the presence or absence of AMF structures is counted each time a root intersects with the gridline. The presence of an AMF structure at the intersect of root and gridline is counted as a positive event, while the absence of an AMF structure is counted as a negative event. The process is then repeated for the vertical gridlines (Giovannetti and Mosse, 1980).

There are several different measures to quantify colonization using the gridline intersect method. The most common is percent root length colonized (PRLC) (Treseder, 2013). The number of positive intersects are divided by the total number of intersects counted to give an estimate of PRLC. In the majority of studies, researchers count fungal structures including hyphae, arbuscules and vesicles. Some researchers, however, have measured arbuscules only as this is the main site of nutrient exchange (Smith and Gianinazzi-Pearson, 1990; Ezawa et al., 2002). Arbuscules only survive for short periods of time (two days) and are more difficult to observe than hyphae, so this method is rarely used (Treseder, 2013).

The extent to which greater PRLC results in increased nutrient transfer to the host is still a topic of debate amongst researchers. Although a meta-analysis found that the response ratio of phosphorus content and plant biomass rose significantly and exponentially with increasing PRLC, responses varied widely by AMF taxa and plant species (Treseder, 2013). Extraradicle hyphae in the soil are responsible for a large portion of nutrient uptake by AMF, and some genera, such as *Gigaspora* and *Scutellospora*, produce more extensive extraradicle hyphae networks (Hart et al., 2002). As such, hyphal length in soils is often used as an indicator of AMF biomass (Bardgett, 1991; Sylvia, 1992).

Some researchers have measured total root length colonized (TRLC) as opposed to PRLC (Treseder, 2013). This can be done on a per plant basis or a ground area basis (Nadian et al., 1997). Presence of AMF is still assessed visually but the root length must be determined and the entire root assessed, so this method is much more time consuming than PRLC (Treseder, 2013). Total root length colonized, however, is a more accurate method of estimation than PRLC.

2.3.6.2 Other Methods of Quantification

The visual method is another method of quantifying colonization in stained roots. Roots are placed in a Petri dish, but rather than following a gridline, the researcher simply estimates the overall percentage of the cortex infected (Giovannetti and Mosse, 1980). This method is more subjective than the gridline intersect method, although roots can be moved around at random and counted again, with the results being pooled in order to increase accuracy.

The slide method involves placing roots on a microscopic slide rather than a Petri dish. Ten roots, approximately one centimetre long each, are placed on a slide and the length of cortex infected is measured. This is then expressed as a percentage (Giovannetti and Mosse, 1980). Alternatively, each root segment can be counted as either containing AMF or not containing AMF, and those numbers are summed for a total colonization count.

Although root staining remains the most common method of assessing AMF colonization, several other assessment methods have been used. Several researchers have used quantitative polymerase chain reaction (PCR) of AMF specific DNA to estimate AMF presence (Filion et al., 2003; Alkan et al., 2004). This technique is expensive and

requires a high level of expertise, and as such is much less common than PRLC (Treseder, 2013). Some researchers have also quantified AMF by measuring characteristic biochemical markers. The cost and time requirements for these methods, however, render them impractical for routine use (Vierheilig et al., 2005).

2.3.7 Arbuscular Myccorhizal Fungi Summary

Arbuscular mycorrhizal fungi play an important role in phosphorus nutrition for many plants. In certain environments they can provide phosphorus and other nutrients that would otherwise be limiting. Crop rotation affects AMF colonization, with non-mycorrhizal crops decreasing AMF colonization of subsequent crops. This is an important consideration when designing crop sequences to optimize the role of AMF in cropping systems.

2.4 Biological Nitrogen Fixation by *Bradyrhizobium japonicum*

2.4.1 Introduction

The ability of a crop to access sufficient nitrogen for proper growth and yield is one of the most critical aspects of crop production. Crop producers can meet a plants nitrogen requirements through synthetic fertilization or biological nitrogen fixation. The high cost and potential environmental impact of synthetic nitrogen fertilizers makes biological nitrogen fixation an important resource for soybean growers, both agronomically and economically. Worldwide, nitrogen fixation processes produce approximately 200 million tons of fixed nitrogen annually, about half of the nitrogen introduced into the environment (Herridge et al., 2008). Crop rotation affects how nitrogen fixing organisms such as *Bradyrhizobium* interact with a crop and with other soil

biota such as AMF. This affects the degree to which they are able to successfully carry out nitrogen fixation.

Bradyrhizobium japonicum is a legume root-nodulating, nitrogen fixing bacterium that forms a symbiotic relationship with the soybean plant. The bacteria provides the plant with nitrogen fixed from the atmosphere in exchange for plant carbohydrates (Mylona et al., 1995). Although other genera of rhizobia are capable of infecting soybean (Albareda et al., 2009), *Bradyrhizobium japonicum* remains by far the most common source of inoculant. *Bradyrhizobium* inoculant is used to ensure sufficient infection of the soybean plant, particularly in fields that do not have a history of soybeans. Fields with a history of soybean cropping have populations of *Bradyrhizobium* present in the soil (Triplett et al., 1993).

Although unreactive atmospheric nitrogen in the form of N_2 makes up 79 percent of dry air, it is unavailable to plants in this form. Rhizobia bacteria cleave the triple bond in the N_2 molecule and convert nitrogen into a mineral form that is useful for plants (Collino et al., 2015). Nitrogenous compounds are a major limiting factor in plant growth, and nitrogen fixation is a key source of nitrogen acquisition. In soybean, 50-60 percent of the plants nitrogen needs are supplied by biological fixation (Salvagiotti et al., 2008). In high yield crops, the percentage of nitrogen derived biologically can be even higher (Collino et al., 2015). The remaining nitrogen is derived from residual soil nitrogen and mineralized organic nitrogen.

2.4.2 Relationship with Plants

2.4.2.1 Mechanisms of Infection

The establishment of symbiosis is a complex process that involves a host of signalling molecules from both the host legume and the rhizobia bacteria (Janczarek et al., 2015). The infection process is highly species specific (Janczarek et al., 2015). The plant synthesizes and secretes flavonoids (a group of plant metabolites) that when detected by rhizobia result in the bacteria synthesizing and secreting a bacterial chitooligosaccharide signal, known as a nodulation (nod) factor (Oldroyd and Downie, 2008). These nod factors activate nodulation in legumes. In soybeans, a group of flavonoid compounds known as isoflavones (daidzein and genistein) are the major inducers of *Bradyrhizobium japonicum* nod gene expression (Banfalvi et al., 1988). Additions and modifications to the basic chitin backbone of these molecules are important in defining specificity of interactions between rhizobia and host legumes (Dénarié et al., 1996).

Infection of the plant root begins when rhizobia adhere to the epidermal root hairs of the plant (Geurts and Bisseling, 2002). The rhizobia then induce deformation and curling in the root hair cells by reinitiating tip growth in an inward direction, allowing the bacterium to become embedded in a cavity of the curl (Heidstra et al., 1994). From this point a small rhizobial community begins to form. As localized degradation of the cell wall begins a tube-like structure known as the infection thread forms. The infection thread contains rhizobia and extends until it reaches the nodule primordium, where rhizobia are released into cells and begin to differentiate into bacteroids (Haag et al., 2013). Once inside the nodules, these bacteroids begin the nitrogen fixation process.

2.4.2.2 Nutrient Exchange

The specificity of *Bradyrhizobium japonicum* to the soybean plant ensures that *Bradyrhizobium japonicum* inoculates only soybean plants and not other plants in the agroecosystem (Seneviratne et al., 2000). The first root nodules begin to form one week after seeding (Ruark, 2009). Active nitrogen fixation begins at the early vegetative stage and reaches a peak at the mid reproductive stages (Ruark, 2009). Nodules will fix nitrogen for six to seven weeks, at which point they will begin to senesce (Conley and Christmas, 2005).

Rhizobia bacteroids provide nitrogen to the host plant by reducing atmospheric N_2 to ammonium using the enzymatic complex of nitrogenase (Gibson et al., 2008). This is an energy intensive process that requires significant carbon inputs from the host plant. The anaerobic compartment that is formed by the nodule prevents oxygen from inactivating the nitrogenase enzyme via the presence of leghemoglobin, which binds to oxygen (Janczarek et al., 2015). Uptake of ammonium by the bacteroids themselves is rendered inoperative when rhizobia is in the symbiotic state with a host, although free-living rhizobia can take up ammonium under starvation conditions (Dilworth and Glenn, 1982). The reduction of dinitrogen is characterised in Equation 1:

$$N_2 + 8H^+ + 8e- + 16ATP \rightarrow 2NH4^+ + H_2 + 16ADP + 16P_i \text{ [Equation 1]}$$

The next step of the fixation process is ammonium transport. Ammonium is likely transported from bacteria cytoplasm to plant cytoplasm via membrane diffusion (Lodwig and Poole, 2003). Plant-derived membranes known as the peribacteroid membrane form the exchange interface between plant and rhizobia bacteroid (Mylona et al., 1995). This structure surrounds the microsymbiont and prevents a defense response

from the plant against the bacteroid (Nap and Bisseling, 1990). A large quantity of ammonium assimilating enzymes in the host creates a concentration gradient between bacteria and host cytoplasm, indicating that the transfer of ammonium could be via diffusion, at least from the bacteroid membrane to the peribacteroid space (Dilworth and Glenn, 1982). Some evidence suggests there may be at least some active transportation via a proton pumping ATPase (Blumwald et al., 1985). This acidifies the peribacteroid space, encouraging NH_4^+ formation from NH_3 and a concentration gradient for diffusion of NH_3 into the peribacteroid space from the bacteroid (Lodwig and Poole, 2003). NH_4^+ may then move to the plant cytosol via an ion channel on the peribacteroid membrane, although this has not been proven (Lodwig and Poole, 2003). Once in the plant cytoplasm ammonium is converted primarily to glutamine via glutamine synthetase and glutamine synthase (Lodwig and Poole, 2003). Soybeans and other tropical legumes then convert glutamine to ureides such as allantoin and allantoin acid (Schubert, 1986).

Carbon is transported from the leaves of the plant to the nodules via the phloem in the form of sucrose, which is degraded by sucrose synthase into UDP-glucose and fructose and introduced into the nodule metabolism (Hawker, 1985). Dicarboxylates are the primary carbon source for bacteroids and are essential for nitrogen fixation. Dicarboxylates are transported at high rates across the peribacteroid membrane, while passive movement of sugars and amino acids occurs at moderate rates but not enough to support nitrogenase activity. This supports the notion that dicarboxylates are the main source of nitrogenase activity in the bacteroid (Udvardi et al., 1990).

2.4.3 Factors Affecting Nitrogen Fixation

2.4.3.1 Inoculation

Soybean inoculants are products containing *Bradyrhizobium japonicum* that are applied to or with the seed at planting. Inoculants ensure there are adequate populations of *Bradyrhizobium* present in the rhizosphere to ensure proper nodulation and BNF. The majority of commercial inoculants are in the form of a liquid that is applied to the seed, or a granule that is seeded with the seed. Numerous studies have shown that inoculated soybeans produce higher seed yield, seed nitrogen content, plant chlorophyll content and plant biomass (Albareda et al., 2009). Even in situations where inoculation does not translate into an increase in yield, nitrogen concentration of the plant or seed is often higher than non-inoculated plants (Wani et al., 1995).

Rhizobia populations can persist in soil for years (Triplett et al., 1993). These populations may consist of native strains or naturalized strains (Vessey, 2003). Native strains are indigenous strains that have nodulated wild legumes. They may also be capable of nodulating introduced crops, however these strains may be less effective than commercial inoculant strains that have been selected specifically for high levels of nodulation (Deaker et al., 2004). Naturalized strains are those that are present in the soil due to inoculation from a previous legume crop. Some studies have suggested that inoculating a field using a commercial inoculant despite the presence of native strains may be economical due to the fact that native strains are often less effective fixers of nitrogen than commercial strains (Deaker et al., 2004). Strains that are adapted to certain climatic conditions will result in more nodulation when soybeans are grown in those conditions. Lynch and Smith (1993) found that rhizobial strains isolated from cool soils

in Japan produced more root nodulation and greater yield in soybeans grown in cool conditions compared to commercial inoculants.

Inoculation is most important in fields without a history of soybean, as these fields often do not have enough natural populations of *Bradyrhizobium* present to support significant BNF (Hassen et al., 2014). Bergersen et al. (1989) applied inoculant at four rates to soybeans in a field with no history of soybean or inoculant use. They found that the highest rate of inoculation resulted in the highest rate of nitrogen fixation, seed nitrogen content, and seed yield. Increased inoculation also resulted in more uniform nodulation and decreased variance of parameters studied. Fields with a history of soybean can suffer declining *Bradyrhizobium* populations over time. Inoculation is often recommended after three to five years without a soybean crop as *Rhizobial* populations in the soil decline after several years of a non-host crop (Pedersen, 2008).

Inoculation is less important in fields with a history of soybean production. Inoculation of crops builds up a naturalized population of rhizobia that, under appropriate conditions, can survive for many years (Thompson et al., 1991). de Bruin et al. (2010) tested 51 inoculant products in 73 fields with a history of soybean across the Midwestern United States. They found that in 63 fields there was no yield response to inoculation compared to an untreated control. Trials conducted in Ontario found a 0.1 t/ha yield increase when inoculating soybeans planted on fields that had a history of well-nodulating soybeans (OMAFRA, 2009).

2.4.3.2 Soil Nitrogen

Biological nitrogen fixation declines as soil nitrogen levels increase. Rhizobia are highly sensitive to the presence of nitrates near root nodules, and decrease nitrogen

fixating activity as the presence of nitrates increases (Vessey and Waterer, 1992). Soil NO_3^- inhibits the functioning of nitrogenase and legheamoglobin, which inhibits nodule formation (Zahran, 1999). The presence of soil nitrates may also affect yield. Hassen et al. (2014) found that fields with lower nitrate levels produced higher soybean yields than those with high nitrate levels.

Brockwell et al. (1989) compared soybean grown on previously cropped and fallow soils at different rates of inoculation. Although initial soil nitrogen and plant growth rates were lower on previously cropped soils, nitrogen fixation was higher, likely as a result of lower mineral nitrogen. Although rates of N_2 fixation declined with increasing mineral nitrogen, this was offset somewhat by increasing the rate of inoculation. This indicates that the negative effect of mineral nitrogen on nitrogen fixation can be offset, to some degree, by increasing inoculation rates.

Super-nodulating varieties that produce more nodules than standard conventional varieties may be less susceptible to the presence of nitrates. Song et al. (1995) compared conventional varieties, mutants that produce twice as many nodules as their conventional checks, and mutants that produce six times as many nodules. They found that adding fertilizer nitrogen reduced nodule weight of conventional varieties by 65 percent, the 2x phenotype by 53 percent, and increased nodule weight in the 6x variety by 7 percent. Addition of fertilizer nitrogen reduced the number of nodules by 32 percent in conventional varieties, 33 percent in the 2x phenotype, and 22 percent in the 6x phenotype. The 2x and 6x phenotype soybeans fixed 13-21 percent more nitrogen than cv. Centaur, as estimated by the xylem ureide technique. The 6x phenotype yielded significantly less than the conventional varieties, likely due to a parasitic effect of

nodules on the soybean plant at such high levels of nodulation (Song et al., 1995). Most studies have shown that nitrogen fertilizer provides no significant yield benefit to soybean even at high amounts of application (Freeborn et al., 2001; Gan et al., 2003). Some studies, however, have shown that nitrogen fertilizer can positively impact yield at very high levels of application (Purcell et al., 2004; Ray et al., 2006). The high cost, however, precludes this management strategy in most situations.

2.4.3.3 Crop Rotation

Crop rotation can affect *Bradyrhizobium japonicum* and the degree of nodulation. As soil nitrate is a potent inhibitor of N_2 fixation, crops that leave more nitrogen in the soil tend to result in decreased N_2 fixation the following year (Peoples et al., 1995b). Bergersen et al. (1989) found that soybean grown on a cereal crop fixed greater amounts of nitrogen and contributed more fixed nitrogen to the soil than soybean grown on fallow. This was due to the higher level of soil nitrates after fallow.

Crop rotation is an important factor in determining how much nitrogen will be contributed to or removed from the soil. Peoples et al. (1995) found that only 11 of 33 soybean crops grown in a trial in Australia contributed more nitrogen to the soil than was taken up by the seed. All of the rotations that did have a net return of nitrogen following soybean were rotations that featured several cereal crops or a double wheat crop, leaving soil nitrate levels low and allowing for increased N_2 fixation. Conversely, N_2 fixation was lower and nitrogen benefit lower at sites where soybean was grown after two or three years of grain legume. N_2 fixation was also decreased in fields that had a history of pasture legume or where additional nitrogen had been added. In intercropping systems, competition for nitrogen from cereal crops can promote N_2 fixation in legumes (Chandel

et al., 1989). Multiple regression analysis has shown a high correlation ($R^2 = 0.80$) between N_2 fixation, *Bradyrhizobium japonicum* numbers, and soil nitrate levels, suggesting all three are interrelated (Peoples and Herridge, 1990). *Bradyrhizobium* numbers and N_2 fixation both increase as soil nitrate levels decline. Various crop rotations can also affect N_2 fixation through their impacts on legume growth.

Crop rotation affects *Bradyrhizobium japonicum* populations less than it affects AMF colonization. This is especially true in fields with a history of soybean production. Triplett et al. (1993) found that *Bradyrhizobium japonicum* numbers did not decline in a long term corn-soybean rotation unless the field was planted to continuous corn for more than four years. Other studies confirm that *Bradyrhizobium japonicum* is persistent in the soil for several years without its host (Kamicker and Brill, 1987; Brunel et al., 1988). Hiltbold et al. (1985), however, found that *Bradyrhizobium japonicum* declined rapidly when cotton or corn was planted, though non-host legumes did not affect *Bradyrhizobia* numbers. This persistence is in significant contrast with certain other rhizobial species such as *Rhizobium meliloti*, whose population was significantly influenced by the number of years since alfalfa had been present in a field (Triplett et al., 1993).

2.4.3.4 Environmental Factors

Bradyrhizobium japonicum is affected by temperature and thrives in ideal temperatures. Ideal temperature for *Bradyrhizobium japonicum* ranges from 25°C to 30°C (Deaker et al., 2004). At temperatures above this ideal range rhizobia quickly begin to die. Brockwell et al. (1987) found that less than 0.2 percent of rhizobia survived longer than 24 hours at a temperature of 38°C. Ideal soil pH is between 6.0 and 7.0. Acid soil pH can have a detrimental effect on *Bradyrhizobium* and nodules often fail to

nodulate in strongly acidic soils (Hassen et al., 2014). Alkaline soils with a pH above 8.0 can also reduce *Bradyrhizobium* populations to the point where inoculation is necessary each time a soybean crop is seeded, even if the field has a history of soybean cropping (Peoples et al., 1995a).

Populations of *Bradyrhizobium* decline faster in sandy soil environments. The lack of organic moisture results in less water holding capacity, causing the bacteria to dry out (Deaker et al., 2004). Water stress affects root nodules negatively, disrupting the mechanisms for oxygen control in nodules, resulting in reduced activity and nodule senescence (Sprent, 1972). Nodules can also be affected negatively when water levels are above field capacity (Sprent, 1972). Factors that affect the viability of the host legume, such as nutrient deficiency, disease, soil pH and mineral toxicity, will in turn have a detrimental effect on the ability of *Bradyrhizobium* to effectively nodulate soybean (Peoples et al., 1995b).

Seed coat toxicity has also been identified as a factor affecting the survivability of rhizobia after is applied to the seed (Deaker et al., 2004). Soybean seeds contain toxins that are inhibitory to the growth of *Bradyrhizobium*. Polyphenolic aglycone compounds in root diffusates of soybean have been identified as being toxic to *Bradyrhizobium* (D'Arcy-Lameta, 1986). Damage to *Bradyrhizobium* proteins can also occur through metal-catalyzed (Fe_3^+) oxidation reactions (Deaker et al., 2004). Damage to *Bradyrhizobium* DNA molecules can occur when exposed to O_2^- and H_2O_2 (Deaker et al., 2004).

2.4.3.5 Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi are an important source of plant phosphorus, particularly in phosphorus deficient soils. Phosphate deficiency can also limit nitrogen fixation in legumes, although this phenomenon is most pronounced in acidic tropical and subtropical soils (Mullen et al., 1988). The ability of nitrogenase to reduce dinitrogen to ammonia is dependent on ATP. For every mol of N_2 that is fixed, 21 mol of ATP are consumed (Salsac et al., 1984). This results in a significant expenditure of phosphorus. Although ATP breaks down into ADP, which can then be converted back into ATP, a steady supply of new phosphorus is required. An inadequate supply of phosphorus results in decreased photosynthesis, root growth, nodule formation, translocation of sugars, bacterial density and amount of nitrogen assimilated per unit weight of nodules (Chaudhary et al., 2008). As the plant grows, its energy and phosphorus needs grow as well.

The response of rhizobia to phosphorus deficiency is highly dependent on strain. Cassman et al. (1981) found that two strains of rhizobia fixed nitrogen just as efficiently at a very low concentration of phosphorus (0.05 µm) as at a high concentration of phosphorus (2000 µm). Another strain struggled to survive at phosphorus concentrations below 1 µm, suggesting this species would perform poorly in low phosphorus environments.

Piccini et al. (1988) studied alfalfa plants inoculated with rhizobium, AMF, or both. They found that plants inoculated with both AMF and rhizobium had greater shoot dry weight, oxygen uptake and nodule nitrogenase activity than plants inoculated with just one symbiont. Plants inoculated with both also had the lowest root to shoot ratio.

This indicates that there is strong competition for photosynthates, and increased shoot growth may come at the expense of decreased root growth. Kucey and Paul (1982) found that faba beans inoculated with both AMF and rhizobium fixed more nitrogen than those inoculated with only one of these symbionts. They attributed this increase to an increase in nodule biomass.

There is evidence that some soybean lines may be dependent on AMF in order to successfully fix nitrogen. Nwoko and Sanginga (1999) tested ten soybean lines for the dependency of *Bradyrhizobium* to fix nitrogen based on the presence of AMF. They found one soybean line was highly dependent on AMF for N_2 fixation (>30 percent dependence). One line was moderately dependent on AMF (10-30 percent dependence). Five of the ten lines were not dependent on AMF for N_2 fixation. Mycorrhizal dependence was present despite mycorrhizal colonization ranging from 16-33 percent, while some studies have shown much higher levels of mycorrhizal colonization in soybean (Khalil et al., 1993). Khalil et al. (1993) found that soybean had higher mycorrhizal dependency than corn, but the degree of dependency varied by cultivar. He found that two of three unimproved (wildtype) soybean cultivars benefited vastly from the presence of AMF, while two improved cultivars showed considerably less dependency. This suggests that new cultivars may be less dependent on AMF than previous ones.

Mycorrhizae may prevent root nodule senescence when the soybean plant is under drought stress. Premature senescence of rood nodules is a common symptom of drought stress in soybeans. Ruiz-Lozano et al. (2001) found that soybean plants inoculated with *Bradyrhizobium japonicum* and *Glomus mosseae* increased protein content by 15 percent,

leghemoglobin content by 25 percent and acetlyene reductase activity (a measure of nitrogenase activity) by 112 percent compared to soybean plants that had only been inoculated with rhizobium. This suggests that AMF may be particularly important to N_2 fixation when the plant is under stress.

2.4.4 Assessing Biological Nitrogen Fixation

There are a variety of methods available to estimate the percentage of nitrogen in a plant that has been fixed biologically. They vary greatly in complexity, methodology, and accuracy. Some of the common methods include the nitrogen balance method, the nitrogen difference method, the ureide (nitrogen solute) method, the acetylene reduction method, and the ^{15}N natural abundance method (Unkovich et al., 2008). The focus of this review will be the ^{15}N natural abundance method.

2.4.4.1 ^{15}N Natural Abundance Method

The ^{15}N natural abundance method utilizes measurements of ^{15}N, a stable and naturally occurring isotope of nitrogen that contains an extra neutron, to estimate the percent of nitrogen in a plant that has been derived from atmospheric N_2 (%Ndfa). The most common isotope of nitrogen, ^{14}N has an atomic mass of 14, and the ^{15}N isotope of the nitrogen molecule is much less abundant than ^{14}N. The abundance of this isotope is expressed as a proportion of total nitrogen (atom% ^{15}N). ^{15}N is present in the atmosphere at a proportion of 0.3663 atom%. This rate is generally consistent in the atmosphere around the world, which makes it a useful measurement tool (Hogberg, 1997). Proportions of ^{15}N in soil, however, are often slightly higher than the value found in the atmosphere. This is a result of the fractionation that occurs in soil nitrogen transformations (Unkovich et al., 2008). By calculating the difference in ^{15}N between a

soil sample and the atmospheric proportion, a $\delta^{15}N$ (delta ^{15}N) value can then be determined, which can then be used to estimate %Ndfa. $\delta^{15}N$ is expressed in permils (‰). See Equation 2 for the $\delta^{15}N$ calculation:

$\delta^{15}N(‰) = ((\text{sample atom\%}^{15}N - 0.3663) / 0.3663) \text{ X } 1000$ [Equation 2]

For example, a sample with an atom%^{15}N of 0.4 would be calculated as follows (Equation 3):

$\delta^{15}N(‰) = ((0.4 - 0.3663) / 0.3663) \text{ X } 1000 = +92‰$ [Equation 3]

A plant that derives all of its nitrogen from atmospheric N_2 fixation would be expected to have a $\delta^{15}N$ value very close to that of the atmosphere (0.3663 atom%). A plant that derives all of its nitrogen from the surrounding soil would be expected that have a $\delta^{15}N$ value very close to that of the soil. By comparing the $\delta^{15}N$ value of a plant sample to that of the soil, we can estimate what percentage of nitrogen was derived from soil nitrogen sources and what was derived atmospherically (via fixation). The $\delta^{15}N$ value of the plant sample should lie somewhere between that of soil N and the atmospheric rate. See equation 4:

%Ndfa = (($\delta^{15}N$ of soil N - $\delta^{15}N$ of N_2 fixing legume) / ($\delta^{15}N$ of soil N - $\delta^{15}N$ of N_2)) X 100 [Equation 4]

Measuring the $\delta^{15}N$ of soil N is technically difficult and inconvenient, so a non-N_2 fixing reference plant is often used instead. This is often done in field experiments by planting a non N_2 fixing crop, such as canola, next to the N_2 fixing crop. The formula remains the same, with the $\delta^{15}N$ of the reference plant replacing that of soil N (Equation 5):

%Ndfa = ((δ^{15}N of reference plant - δ^{15}N of N_2 fixing legume) / (δ^{15}N of reference plant - δ^{15}N of N_2)) X 100 [Equation 5]

2.4.4.2 Advantages and Limitations of the ^{15}N Natural Abundance Method

The technique has a variety of advantages over other methods of measuring N_2 fixation. It can be applied anywhere that a N_2 fixing and non-N_2 fixing plant can be grown together. This allows experiments to be readily conducted in the field, which is often expensive or impractical for other methods of determining N_2 fixation (Unkovich et al., 2008). There are, however, several limitations to the technique. The natural variability of nitrogen in the soil means that reference plants may not be taking nitrogen from an identical pool as the N_2 fixing plant. This may reduce accuracy. In field experiments, it is more common for researchers to harvest shoots only, instead of the whole plant. In this case it is important for researchers to adjust for isotopic fractionation within the plant (Unkovich et al., 2008). This can be accounted for using a correction known as a B value. There are published guidelines on the correct B value to use for specific legumes and regions (Unkovich et al., 2008). The B value has little effect on %Ndfa calculations when N_2 fixation is low, but can become much higher when there is a significant level of N_2 fixation (Unkovich et al., 2008). Incorporating the B value would alter the %Ndfa calculation as such (Equation 6):

%Ndfa = ((δ^{15}N of reference plant - δ^{15}N of N_2 fixing legume) / (δ^{15}N of reference plant – B value)) X 100 [Equation 6]

Another limitation of the ^{15}N natural abundance method is that non-N_2-fixing reference plants do not necessarily take up nitrogen in the same manner as their N_2-fixing counterparts. This can lead to inaccuracies when estimating N_2 fixation (George et al.,

1993). Soybean mutant isolines that do not nodulate can reduce inaccuracy when estimating N_2 fixation as they behave similarly to their N_2-fixing soybean counterparts, with the exception that they do not nodulate. Using non nodulating mutant isolines is particularly useful for reducing inaccuracy when levels of N_2 fixation are high (George et al., 1993).

2.4.5 Biological Nitrogen Fixation Summary

Biological nitrogen fixation is an important factor in nutrient acquisition for a variety of crops grown on the Northern Great Plains. It is because of this process that legumes are such a valuable addition to crop rotations on the prairies. Understanding how various factors affect biological nitrogen fixation will help producers make more informed decisions to maximize agronomic efficiency.

2.5 Conclusion

The importance of crop rotation in the management of agricultural systems has made it a frequent and widespread area of research. More than a century of research and thousands of scientific papers have shown that crop rotation can significantly affect crop yield, as well as a variety of other agronomic and ecological factors, such as AMF colonization and nitrogen fixation. The ability of AMF and *Bradyrhizobium* to improve crop growth and yield has also been well documented. Crop rotation is clearly an important factor in their successful contribution to plant growth and yield, although considerably more so for AMF.

With the large scale expansion of soybean production into Manitoba, there are a number of research questions that have yet to be answered. Determining how soybean best fits into current rotational systems in Manitoba will require short and long term rotational experiments. The role of AMF in phosphorus acquisition, its potential benefit to yield, and its relationship with *Bradyrhizobium*, while documented elsewhere, are not well understood on the Northern Great Plains. The degree of biological N_2 fixation in soybean, and the impact of crop rotation on this process, has not been properly studied in Manitoba. Improving understanding in these areas of research will be important to advancing soybean production in Manitoba.

Several hypotheses were made for this experiment. We hypothesized that:

i) Soybean grown on soybean as the preceding crop would result in lower soybean grain yield as a result of increased disease pressure.

ii) Soybean grown on canola as the preceding crop would result in lower soybean grain yield as a result of competition from herbicide resistant canola volunteers.

iii) Soybean grown on canola as the preceding crop would result in reduced mycorrhizal colonization by arbuscular mycorrhizal fungi as canola does not readily form the symbiotic relationship with AMF.

iv) Increased AMF colonization of the soybean roots would stimulate and increase BNF through greater nutrient and water uptake.

3.0 MATERIALS AND METHODS

3.1 Site Description

Experiments were located at the Ian N. Morrison Research Farm in Carman, Manitoba (49°29’59.0”N, 98°01’50.4W”), Kelburn Farms in Glenlea, Manitoba (49°41’46.6”N, 97°06’54.5”W) , and the Canada-Manitoba Crop Diversification Centre in Portage la Prairie, Manitoba (49°57’32.4”N, 98°16’32.6”W). The soil at Carman was an Orthic Black Chernozem clay loam soil of the Eigenhof series (Mills and Haluschak, 1993). The soil at Portage la Prairie was a Gleyed Rego Black Chernozem silty clay loam soil of the Gnadenthal-Neuhorst complex. The soil at Kelburn was a Gleyed Black Chernozem clay soil of the Scanterbury series (Agriculture and Agri-Food Canada, 1999). The sites at Carman and Portage had been previously cropped to flax, while sites at Kelburn had been previously cropped to spring wheat. Climate data was obtained from weather stations in Carman, Portage la Prairie, St. Adolphe and Winnipeg (James Richardson International Airport) as they were the closest stations to the sites (Table 3.1). Long term averages were obtained from Carman, Portage la Prairie and Winnipeg, as no long term data was available from the St. Adolphe station. In 2013, precipitation was lower than the long-term average (LTA) at Carman (86% of LTA) and about average at Kelburn (103% of LTA). In 2014, precipitation was average at Carman (99% of LTA), higher than average at Kelburn (117% of LTA), and average at Portage (101% of LTA).

Table 3.1. Mean monthly, growing season, and long term average (LTA) (1981-2010) temperature and total precipitation at Carman, Kelburn and Portage research sites (Government of Canada, 2015; MAFRD, 2015).

Research Site	May	June	July	August	September	October	Growing Season
Temperature				°C			
Carman 2013	10.5	17.7	18.7	18.9	15.1	6.2	16.2
Carman 2014	11.3	16.6	18.2	18.7	13.1	7.7	15.6
Carman LTA	11.6	17.2	19.4	18.5	13.4	5.4	16.0
Kelburn 2013	11.6	18.4	19.3	19.8	16.1	5.0	17.0
Kelburn 2014	12.0	17.4	18.5	19.4	14.0	7.5	16.3
Kelburn LTA	11.6	17.0	19.7	18.8	12.7	5.0	16.0
Portage 2013	10.9	17.8	19.2	19.5	15.4	6.3	16.6
Portage 2014	11.3	16.8	19.0	19.2	13.4	8.1	15.9
Portage LTA	11.9	17.1	19.3	18.0	12.5	6.1	15.8
Total precipitation				mm			
Carman 2013	116.0	50.6	49.2	59.7	31.0	13.2	306.5
Carman 2014	30.9	116.7	47.5	122.4	46.6	4.6	364.1
Carman LTA	69.6	96.4	78.6	74.8	49.0	43.4	368.4
Kelburn 2013	87.3	60.8	90.3	75.4	33.1	14.5	346.9
Kelburn 2014	66.8	157.1	40.3	91.8	24.8	4.7	380.8
Kelburn LTA	56.7	90.0	79.5	77.0	45.8	37.5	349.0
Portage 2013	90.6	68.6	99.8	65.2	39.4	36.6	363.6
Portage 2014	49.0	135.3	20.2	92.3	45.6	3.2	342.4
Portage LTA	58.4	90.0	78.4	68.3	50.1	43.2	345.2

3.2 Experimental Design and Treatments

The experiment was designed as a randomized complete block design (RCBD) with four replicates per site. Five field trials were conducted from 2012 to 2014. Each experimental trial consisted of a two-year crop sequence. Two experiments were conducted from 2012-2013 and three experiments from 2013-2014. The first year of each experiment consisted of a treatment crop; canola, corn, soybean and spring wheat, and the second year was planted to soybean.

3.3 Experimental Management

3.3.1 Seeding

Spring wheat, canola and soybeans were planted with a disc drill while corn was seeded with a corn planter. At Carman, canola, soybeans and wheat were seeded at a row spacing of 19 cm. Corn rows were spaced 80 cm apart. At Kelburn, canola, soybeans and wheat were seeded at a row spacing of 19 cm, corn rows were spaced 76 cm apart. At Portage, canola, soybeans and wheat were seeded at a row spacing of 15 cm with corn rows spaced 80 cm apart. Seeding rates were the same at all site years, except at Kelburn in 2013 where a seeding rate error occurred. Canola was seeded at a rate of 1 100 000 seeds ha^{-1} (1 435 000 seeds ha^{-1} at Kelburn in 2013). Corn was seeded at a rate of 69 000 seeds ha^{-1}. Soybean was seeded at a rate of 540 000 seeds ha^{-1}(675 000 seeds ha^{-1} at Kelburn in 2013). Wheat was seeded at a rate of 311 000 seeds ha^{-1}(379 000 seeds ha^{-1} at Kelburn in 2013).

The canola variety planted was 73-75 RR. The corn variety planted was DKC 26-79 RR. The wheat variety planted was Glenn. The soybean variety planted was 25-10

RR at all site years in 2012, and 24-10 RR at all site years in 2013. Seeding rates and dates were based on recommendations from Manitoba Department of Agriculture (MDA) (Table 3.2). Seeding rates were slightly higher at Kelburn in 2013 due to a seeding rate error. Plot sizes varied slightly by location. Plots were 8m by 6.5m at Carman, 8m by 6m at Kelburn, and 8m by 7.5m at Portage. Plots were cultivated with shanks once in the spring and once in the fall after harvest. Corn plots were also disced twice in the fall and once in spring.

Test crop soybeans (24-10 RR) were seeded at rates of between 445 000 and 545 000 plants/ha depending on site year (Table 3.3). A higher seeding rate was used at Carman in 2013, but due to lodging of the soybean plant this seeding rate was reduced in 2014. Test crop soybeans were planted with a disc drill at the same row spacing as treatment crop soybeans. Both treatment and test crop soybeans were inoculated with liquid and granular inoculant. The liquid inoculant was Optimize by Novozymes (minimum 2 billion *Bradyrhizobium japonicum* cells per gram) and the granular inoculant was Cell-Tech West by Novozymes (minimum 1 billion *Bradyrhizobium japonicum* cells per gram). A 2m by 8m strip of canola (73-75 RR seeded at a rate of 1 100 000 seeds ha^{-1}) was seeded at the back portion of each soybean test crop plot in order to compare nitrogen fixation between the two crops.

Table 3.2. Seeding date and harvest date of first year treatment crops at Carman (2012,2013), Kelburn (2012,2013) and Portage (2013).

	Crop			
	Canola	Corn	Soybean	Wheat
Seeding Date				
Carman 2012	4-May	17-May	16-May	4-May
Carman 2013	17-May	10-Jun	23-May	17-May
Kelburn 2012	10-May	15-May	10-May	10-May
Kelburn 2013	29-May	5-Jun	29-May	29-May
Portage 2013	6-Jun	7-Jun	6-Jun	6-Jun
Harvest Date				
Carman 2012	16-Aug	3-Oct	27-Sep	9-Aug
Carman 2013	3-Sep	24-Oct	1-Oct	26-Aug
Kelburn 2012	27-Aug	3-Oct	24-Sep	8-Aug
Kelburn 2013	16-Sep	21-Oct	2-Oct	6-Sep
Portage 2013	17-Sep	29-Oct	9-Oct	17-Sep

Table 3.3. Soybean test crop seeding rates, actual plant stands, seeding dates, and harvest dates at Carman 2013, Kelburn 2013, Carman 2014, Kelburn 2014 and Portage 2014.

Site year	Seeding Rate	Plant Stand	Seeding Date	Harvest Date
	Plants ha^{-1}	Plants ha^{-1}		
Carman 2013	545 000	510 000	22-May	01-Oct
Kelburn 2013	445 000	340 000	05-Jun	02-Oct
Carman 2014	495 000	435 000	26-May	07-Oct
Kelburn 2014	445 000	545 000	30-May	14-Oct
Portage 2014	445 000	600 000	04-Jun	21-Oct

3.3.2 Fertility

Nitrogen, phosphorus and sulfur were applied to the corn, wheat and canola reference crops. Fertilizer rates were based on soil testing levels (Table 3.2) and guidelines based on the Manitoba Soil Fertility Advisory Committee (Table 3.5). Neither treatment nor test crop soybeans received any fertilizer.

Table 3.4. Soil nutrient status of each experimental site in early May before test crop planting. Nitrate was measured to a depth of 0-120 cm, split into four soil zones (0-15 cm, 15-60 cm, 60-90 cm, 90-120 cm). Olsen phosphorus and potassium were measured at a depth of 0-15 cm. Soil pH and sulfur was measured at a depth of 0-60 cm, split into two soil zones (0-15 cm, 15-60cm).

Research Site	Soil Depth	pH	N[1]	P[2]	K	S[3]
	cm		----------ppm----------			
Carman 2013	0-15	6.6	3.3	10.3	287	6.9
	15-60		4.5			7.9
	60-90		2.9			
	90-120		1.8			
Carman 2014	0-15	6.7	5.3	9.3	240	
	15-60	7.7	6			
	60-90		3.6			
	90-120		3.1			
Kelburn 2013	0-15	6.9	10.4	12.9	356	9.5
	15-60		9.9			7.8
	60-90		3.8			
	90-120		3.2			
Kelburn 2014	0-15	7.3	7.9	29.3	367	
	15-60	7.6	6.1			
	60-90		3.1			
	90-120		3			
Portage 2014	0-15	8	9.3	9.8	249	
	15-60	8.1	5.9			
	60-90		3.1			
	90-120		2.4			

[1]nitrate-N, [2]Olsen-P, [3]sulfate-S

Table 3.5. Fertilizer rates applied to first year treatment crops.

Site Year	Crop	Fertilizer Applied		
		Kg ha^{-1}		
		N	P	S
Carman 2012	Canola	100	17	28
	Corn	100	17	0
	Wheat	67	17	0
	Soybean	0	0	0
Kelburn 2012	Canola	100	22	22
	Corn	100	22	0
	Wheat	45	22	0
	Soybean	0	0	0
Carman 2013	Canola	70	15	28
	Corn	70	15	0
	Wheat	34	8	0
	Soybean	0	0	0
Kelburn 2013	Canola	100	22	22
	Corn	100	22	0
	Wheat	45	22	0
	Soybean	0	0	0
Portage 2013	Canola	50	39	28
	Corn	50	39	0
	Wheat	28	22	0
	Soybean	0	0	0

3.3.3 Weed Control

Weeds in both treatment and test crops were controlled using herbicides. The herbicides and rates were the same for each crop type in all years of the study. Soybean and corn were sprayed with glyphosate (Roundup Weathermax) at a dose of 1.66L ha^{-1} (540 g a.e. ha^{-1}). Canola was sprayed with glyphosate at a dose of 0.82 L ha^{-1} (270 g a.e. ha^{-1}). Wheat was sprayed with a mixture of bromoxynil (Buctril M) at a dose of 0.99 L ha^{-1} and pinoxaden (Axial BIA) at a dose of 1.19 L ha^{-1}. Spraying for the first year

treatment crops was done with a handheld boom and backpack sprayer. Spraying for the second year test crops was done with a bicycle sprayer.

Due to deer and rabbit damage a fence was built at Carman in both site years to protect the test crop soybeans. Damage to soybeans before the fence was built at Carman in 2013 from pests was minor. Serious damage to the soybean test crop from pests occurred at Kelburn in 2013 as there was no fence.

3.3.4 Volunteer Soybeans

Volunteer soybeans sometimes emerged in the soybean-soybean crop sequences as a result of harvest losses and pod shatter. In the soybean test crop at Carman and Kelburn in 2013, these volunteers were removed from the plots and weighed for yield separately. Volunteers in 2013 were a different variety (25-10 RR) than the test crop soybeans (24-10 RR) and could be visually distinguished by their darker stem color. Volunteer soybeans in 2014 were removed at the cotyledon stage from the Kelburn site as they emerged before the test crop. No volunteer soybeans were detected at Carman or Portage in 2014.

3.4 Data Collection

3.4.1 Soil Nutrients

Soil samples were collected for nutrient analysis in early May before planting. Three soil samples were taken from each plot with a dutch auger at four depths: 0-15 cm, 15-60 cm, 60-90 cm, and 90-120 cm. Samples were sent to Agvise Laboratories in

Northwood, North Dakota and analyzed for soil nitrate, Olsen P, potassium, sulfur and electrical conductivity (mmhos/cm).

3.4.2 Yield

Both the first year treatment crops and the soybean test crops were harvested for yield. Crops were harvested when they had reached maturity (Table 3.2 and 3.3). Yield samples were taken from the middle 4m of each plot using a plot combine. Samples were processed with a Clipper M2BC seed cleaner (A.T. Ferrell, Bluffton, Indiana, USA). After cleaning samples were weighed and converted to kg ha^{-1}. Sub-samples were taken and dried for 48 hours at 65°C to determine seed moisture content. All sample weights were adjusted to the standard test moisture content of 13 percent. See Table 3.6 for first year treatment crop yield data.

Table 3.6. Yield of first year treatment crops at Carman (2012,2013), Kelburn (2012,2013) and Portage (2014).

	Carman		Kelburn		Portage
Treatment	2012	2013	2012	2013	2013
	---------- Kg ha^{-1} ----------				
Canola	1597	2640	2187	2845	3010
Corn	5521	9481	7549	8783	7713
Soybean	2677	2878	2938	3051	2279
Wheat	4775	5091	3545	2532	5123

3.4.3 Carbon to Nitrogen Ratios

Two samples of one square meter each were hand harvested from the first year treatment crops to determine above ground biomass C:N ratios of the treatment crops. Only shoots were harvested. Samples were threshed by hand using a standard test sieve (W.S. Tyler, Mentor, Ohio, USA). Samples were dried for 48 hours at 65°C then ground

using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Carbon to nitrogen ratio samples were sent to Agvise Laboratories (Northwood, ND, USA) for C:N ratio analysis.

3.4.4 Estimating Mycorrhizal Colonization

Soybean test crop roots were sampled at the V3 growth stage. Three plants were sampled per plot. Roots were collected by cutting the plant at ground level and then removing the roots using a long-handled bulb planter (Faithfull Tools, Norwich, Norfolk, UK). Root samples were then soaked in water for 24 hours and washed using a 2 mm and 500 µm screen to separate the roots from soil and debris. Roots were removed from the screens using fine forceps and stored in centrifuge tubes in an ethanol solution (18 parts ethanol, one part formaldehyde and one part glacial acetic acid) for preservation (McGonigle et al., 1990).

Clearing of roots was required prior to staining for mycorrhizae in roots. This method has been detailed by McGonigle et al. (1990). Roots were placed in a potassium hydroxide solution (nine parts water, one part potassium hydroxide) in order to soften. The roots were then cleared in an autoclave at 121°C and 15 psi for 15 minutes. Samples were then placed in a beaker containing the staining agent, Chlorazol Black E. The solution was 0.13 ml Chlorazol Black E, 150 ml glycerol, 150 ml water, and 150 ml acetic acid. Roots were heated in this solution in an oven for 60 minutes at 90°C, allowing the staining solution to enter the root cortex. The roots were then put in centrifuge tubes containing 50% glycerol and 50% water in order to allow the darkest stains to clear out from the roots.

After one to two weeks of destaining the roots were placed in a glycerol solution on a square Petri dish with grid lines in order to be analysed microscopically. Roots were

spread out evenly across the Petri dish. Roots were then visualized using a dissection microscope (Zeiss International, Oberkochen, Baden-Wurttemberg, Germany) at 200X magnification. The Petri dish was moved horizontally along gridlines with the frequency of a root at 100 gridline intersections observed (McGonigle et al., 1990). Presence or absence of mycorrhizal structures were also recorded for each intersection. A mycorrhizal structure may be hyphae, arbuscules or vesicles. If all horizontal grid lines were counted and 100 independent intersections had not yet been recorded, then the process continued along the vertical gridlines. If there were still not 100 events counted after both the horizontal and vertical gridlines have been counted, the researcher randomly reorganized the roots and started the process along the horizontal gridlines again.

Once at least a hundred intersections were observed, the total number of hyphae, arbuscules, and vesicles was tallied. An intersection where multiple types of mycorrhizal structures were present was tallied as only one mycorrhizal structure. Then the number of negative intersections was subtracted. This number was divided by the total number of events to determine the total percent colonization as shown in Equation 7:

$$\text{Percent Colonization} = \frac{\text{Hyphae+Arbuscules+Vesicles}-\text{Doubles}-\text{Negatives}}{\text{Total Number of Events}} \text{X } 100 \text{ [eqn. 7]}$$

Samples from Carman and Kelburn in 2014 contained spores that were mistakenly identified as vesicles. These spores likely belong to a different fungal species (ie. *Pithium*). The initial count of vesicles in the 2014 samples containing these spores were removed from the entire data set and identified as a negative intersection (no

mycorrhizal activity) as there was generally only a small number (0-10) of actual vesicles per sample.

3.4.5 Estimating Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) of soybean was estimated using the natural abundance method. This method of N analysis compares the $\delta^{15}N$ signature of a nitrogen fixing crop to a non-nitrogen fixing reference crop, from which BNF can be estimated. Strips of canola were seeded at the back of each plot so that both the soybean and canola plants would grow in a similar soil profile. Any soybeans growing in these canola strips were weeded out by hand.

Three soybean plants were sampled from each plot for percent nitrogen derived from fixation (%Ndfa) analysis. In 2013, this was done at the R5 and R6 stages. In 2014, this was done at the V3 and R5 stages. Plant samples were collected by cutting the three soybean plants at ground level and taking the entire above ground portion of the plant. Samples were then dried in an oven for 48 hours at 100°C. The samples were then ground in a Wylie mill (Thomas Scientific, Swedesboro, NJ, USA) and then ground to the consistency of baking flour using a cyclone mill (UDY Corporation, Fort Collins, CO, USA). This homogenized the samples even further so that the small amount of plant material to be used in analysis was representative of the entire plant sample.

In order for the samples to be analyzed by a mass spectrometer, a miniscule amount of plant material was packaged in tin capsules. Samples between 1 and 4 mg (2.5 mg +/- 1.5 mg) were weighed out and placed in the capsules, which were then folded and packed tightly to avoid material loss. The capsules were then placed in Elisha plates (Isomass Scientific, Calgary, AB, Canada) and sent to the Department of Soil Science at

the University of Saskatchewan where they were analyzed for ^{15}N content using a mass spectrometer (253 Ultra High Resolution IRMS, Thermo Fisher Scientific, Waltham, MA, USA). The mass spectrometer determined ^{15}N content via isotopic fractionation, wherein the heavier ^{15}N atoms are separated from other lighter isotopes. Equation 8 was used to determine %Ndfa based on the samples $\delta^{15}N$ signature:

$$\%\text{Ndfa} = \frac{(\delta 15\text{N of reference crop (canola)} - \delta 15\text{N of test crop (soybean)})}{(\delta 15\text{N of reference crop} - \text{B value})} x100 \text{ [Eqn. 8]}$$

Percent Ndfa refers to the percentage of nitrogen in the soybean plant that was fixed biologically. Reference crop and test crop $\delta^{15}N$ refers to the ratio of stable ^{15}N isotopes to ^{14}N isotopes. B value refers to the correction made for within plant fractionation of ^{14}N and ^{15}N. The ^{15}N isotope of nitrogen is found in the atmosphere at a constant rate of 0.3668‰. The ^{15}N isotope is present in soil at a higher portion than in the atmosphere, depending on soil conditions and location. The reference crop is used to estimate the ^{15}N signature of the soil. As canola is a non-nitrogen fixing plant, all of its nitrogen will be derived from the soil and its ^{15}N signature will closely resemble that of the soil. A nitrogen fixing plant will have a lower ^{15}N signature as a significant portion of its nitrogen is derived from atmospheric nitrogen fixed biologically, where the ^{15}N signature is lower. By comparing the difference in values between the $\delta^{15}N$ signature of the two plants, the amount of nitrogen fixed biologically can be estimated. The B value is a correctional value that accounts for nitrogen in the roots, which are typically not sampled. A B value of -1.5 was used in this experiment. This B value was the mean value of seven soybean studies conducted in temperate climate zones reported by Unkovich et al. (2008).

3.4.6 Plant Phosphorus Concentration

Above ground plant samples were taken at the V3 and R5 stages of growth. Three plants were sampled per plot and ground with a Wylie mill (Thomas Scientific, Swedesboro, NJ, USA). Samples were then sent to Agvise Laboratories (Northwood, ND, USA) where it was analysed for total phosphorus content using a digestion mixture. A sample of 1g per plot was analysed for phosphorus concentration. Percent phosphorus was then converted to mg P/kg of plant biomass. Phosphorus content analysis was only conducted in 2014.

3.5 Statistical Analysis

Statistical analysis was conducted using the statistical software program SAS 9.3 (SAS Institute, Carey, NC, USA). Soybean yield, mycorrhizal colonization of soybean roots, and BNF by soybean were the main variables analyzed. Differences in plant populations, soil nitrogen and phosphorus, plant phosphorus content and C:N ratios were also analyzed.

Analysis of variance was conducted with Proc Mixed to test for significant treatment differences. Assumptions of ANOVA were tested using the Shapiro-Wilk test in Proc Univariate to test for normality and by plotting the distribution of residuals to test for heteroscedasticity. Outliers were detected and removed based on studentized residuals (Lund, 1975). Where a significant site year by treatment interaction occurred, treatment results were analysed separately by site year using the SLICE statement of Proc Mixed in SAS. The protected Least Squares Difference was used to determine significant differences ($P < 0.05$).

Regression analysis using Proc Reg was conducted to test for significance of linear and exponential models. Linear regression was conducted for effect of soil phosphorus on mycorrhizal colonization, effect of soil nitrogen on BNF and effect of C:N ratio on soil nitrogen. Correlation analysis using Proc Corr was conducted to test for correlation between mycorrhizal colonization and soybean yield, C:N ratio and BNF, and mycorrhizal colonization and BNF. Exponential regression was conducted for effect of mycorrhizal colonization on total percent plant phosphorus. Relationships between mycorrhizal colonization and BNF, mycorrhizal colonization and yield, mycorrhizal colonization and soil phosphorus, mycorrhizal colonization and plant phosphorus, BNF and soil nitrogen, BNF and C:N ratios, and C:N ratios and soil nitrogen were determined. Individual data points were used in all regression and correlation analysis.

4.0 RESULTS AND DISCUSSION

4.1 Effect of Preceding Crop on Soybean Yield

Soybean yield varied significantly by site year and did not follow a consistent trend over the five site years of the study (Table 4.1, Table 4.2). Results were analyzed by site year as there was a significant site year by treatment interaction effect. When site years were combined there was no significant effect of preceding crop on soybean yield.

Table 4.1. Effect of preceding crop (Crop) and site year (SiteYear) on soybean yield pooled across five site years.

Factor	Treatment	Yield Kg ha^{-1}
Crop	Canola	3000
	Corn	2928
	Soybean	2997
	Wheat	3002
SiteYear	Carman 2013	3774 **a**
	Carman 2014	3093 **b**
	Kelburn 2013	2688 **c**
	Kelburn 2014	2961 **b**
	Portage 2014	2390 **d**
ANOVA		P > F
Crop		0.7901
SiteYear		<.0001
Crop*SiteYear		0.0019

Means within a column followed by a different letter are statistically significant at $P<0.05$ according to Fisher's Protected LSD.

Table 4.2. Effect of preceding crop (Crop) on soybean test crop yield at Carman (2013, 2014), Kelburn (2013, 2014) and Portage (2014).

	Carman		Kelburn		Portage
Treatment	2013	2014	2013	2014	2014
	----------Kg ha^{-1}----------				
Canola	3764 **b**	3095	2844	3110 **a**	2185
Corn	3614 **b**	2995	2529	3042 **a**	2458
Soybean	4158 **a**	3198	2807	2487 **b**	2299
Wheat	3550 **b**	3086	2550	3211 **a**	2619
Mean	3772	3094	2683	2963	2390
P-value					
Crop	0.0084	0.6716	0.1366	0.0004	0.0579

Means within a column followed by a different letter are statistically significant at $P<0.05$ according to Fisher's Protected LSD.

Soybean test crop yield at Carman in 2013 was greater than all other site years. Soybean yield at Carman and Kelburn in 2014 was greater than yield at Kelburn in 2013 and Portage in 2014. Soybean yield at Portage in 2014 was lower than all other site years (Table 4.1). Mean soybean yield for each site was higher than the rural municipality (RM) average at Carman in 2013 and 2014, and at Kelburn in 2014 (Table 7.1). Mean soybean yield was close to the RM average at Kelburn in 2013 and Portage in 2014.

Soybean yield ranged from a high of 4158 kg ha^{-1} for the soybean-soybean sequence at Carman in 2013 to a low of 2185 kg ha^{-1} for the canola-soybean sequence at Portage in 2014 (Table 4.2). Soybean yield varied as a result of treatment differences at some site years, but not others. At Carman in 2013, the soybean-soybean sequence yielded significantly greater than the other three treatments. The soybean-soybean sequence yielded significantly lower than the other three sequences at Kelburn in 2014. There were no significant differences in yield between crop sequences at Carman in 2014, Kelburn in 2013 and Portage in 2014 (Table 4.2).

The corn-soybean and wheat-soybean crop sequences were the most consistent treatments in terms of yield and variability across all five site years. Both sequences yielded greater than soybean-soybean at Kelburn in 2014, and wheat-soybean yielded greater than canola-soybean at Portage in 2014. Both sequences yielded lower than soybean-soybean at Carman in 2013. This was the only site year where corn-soybean and wheat-soybean yielded significantly lower than the other crop sequences. The soybean-soybean sequence showed a high degree of variability, yielding greater than all other crop sequences at one site year and lower than all others at another site year. The corn-soybean and wheat-soybean sequence were relatively stable in terms of yield. Provincial survey data compiled by Manitoba Agricultural Services Coroporation (2012) show that soybean grown on spring wheat and corn yielded 103 and 107 percent of the provincial average soybean yield from 2008 to 2012 (Table 2.2). The survey found that soybean grown on canola yields 101 percent of the provincial average soybean yield, while soybean on soybean yields 95 percent of average soybean yield. This provincial survey data shows that on average, a soybean-soybean sequence yields lower than the provincial average for soybean yield and lower than the other three crop sequences.

The initial hypothesis of this experiment was that the soybean-soybean crop sequence would result in lower yield than other sequences. This was hypothesized due to problems commonly associated with continuous soybean including: a buildup of diseases, weed species shifts, decreasing phosphorus and potassium levels, and decreasing soil organic matter (Schwartz and Steadman, 1978; Bullock, 1992; Rousseau et al., 2007). The soybean-soybean sequence yielded significantly lower than other sequences in only

one site year (Kelburn in 2014). That same sequence yielded significantly higher than other sequences at Carman in 2013.

Several studies have found that growing soybean continuously results in decreased yield, particularly when they are grown over multiple years. Temperly and Borges (2006) conducted a field trial in Wisconsin comparing yield of soybean grown after five years of continuous corn to yield of soybean grown after five years of continuous soybean. They found that soybean grown after continuous corn yielded 42 percent higher in a no-tillage system and 35 percent higher in a conventional tillage system compared to continuous soybean. In another Wisconsin field trial, Meese et al. (1991) found that the yield benefit of soybean following corn was 15 percent compared to continuous soybean. They also found that the yield benefit of soybean following several years of corn was 15 percent higher than soybean rotated annually with corn, indicating that the increase in soybean yield rises the longer soybean is not in the rotation. A study in Northern China found that soybean yield declined 9.9, 13.8, and 19 percent after two, three and four years of continuous soybean production, respectively, compared to soybean rotated annually with corn (Liu and Yu, 2000). Another study in China found soybean yield declined by 18.6 and 35.4 percent after two and three years of continuous soybean compared to soybean rotated with corn (Xu et al., 1999). The declining yield was largely due to the build-up of disease populations over several years, which affects yield through root rot, decreased root branching, decreased nodulation and decreased nitrogen fixation (Liu and Herbert, 2002).

The rotation effect can extend beyond the next year's crop. Porter et al. (1997) found that soybean rotated annually with corn yielded 10 percent higher than continuous

soybean, while a second straight year of soybean after corn yielded 8 percent higher than continuous soybean. This indicates the rotation effect may still be present in the second year soybean crop, resulting in only a minor decrease in yield compared to rotating or alternating crops on an annual basis. Conversely, increasing years of continuous cropping may result in decreasing yield year after year. Crookston et al. (1991) found that a second year of consecutive soybean yielded just as well as soybean rotated annually, while a third year of consecutive soybean showed a significant decrease in yield. This could explain why the soybean-soybean rotation in this study only resulted in lower yield at one site year. While previous research has shown some yield decline after two consecutive years of soybean, the greatest losses in yield occur when soybean is grown consecutively for three or more seasons (Crookston et al., 1991; Porter et al., 1997; Xu et al., 1999; Liu and Yu, 2000). Increasing the length of the study beyond two years would likely have resulted in more significant yield decreases in continuously grown soybean, consistent with previous research where increasing years of continuous soybean resulted in decreasing soybean yields year after year (Crookston et al., 1991; Porter et al., 1997; Xu et al., 1999; Liu and Yu, 2000).

Provincial survey statistics indicate that soybean on soybean results in lower yields compared to soybean grown on a different crop stubble. Manitoba provincial data provided by Manitoba Agriculture Services Corporation (MASC) shows that from 2008-2012 soybean grown on soybean stubble yielded 95 percent relative to the provincial average for soybean yield (Table 2.2). While this study only found significantly lower yield resulting from a soybean-soybean sequence at one site, there was significant variability in yield for the soybean-soybean treatment between site years. The high yield

of the soybean-soybean treatment at Carman in 2013, however was largely due to the presence of volunteer soybeans in those plots and not to any yield benefit conferred by a soybean-soybean sequence.

Considering the current study, more site years were likely needed to determine how factors such as weather and location affect soybean yield. A cool, wet growing season may result in significantly reduced yield in a soybean-soybean sequence compared to other sequences as a result of increased disease incidence. Longer term trials are also needed to determine how the rotation effect changes and affects yield over multiple growing seasons. Previous research has found that yield losses in soybean increase with each successive year of continuous cropping (Crookston et al., 1991; Porter et al., 1997; Xu et al., 1999; Liu and Yu, 2000). It is likely that increasing the length of the continuous soybean sequence would result in increasing yield losses similar to these studies.

An initial hypotheses of the experiment was that the canola-soybean sequence would yield lower than other crop sequences due to the presence of glyphosate resistant volunteer canola. Gulden (2016) found that volunteer canola in soybean can result in soybean yield loss. The persistence of canola in the seed bank and potential for high harvest losses of canola can result in large volunteer populations that compete with soybean for light and nutrients. Volunteer canola pressure was low at all site years, however, and volunteer canola did not seem to have a significant impact on yield. The canola-soybean sequence yielded lower than soybean-soybean at Carman in 2013. There were no other times when the canola-soybean sequence yielded lower than other crop sequences, despite the potential for glyphosate-resistant volunteers in the soybean crop.

Row spacing varied from 15-19 cm depending on site year. The narrow row spacing of soybean in this experiment may have allowed soybean to compete more effectively with volunteer canola than it would at a wider row spacing. This may partially explain why only canola-soybean only yielded lower than one other crop sequences across all five site years.

Volunteer soybeans emerged in several of the soybean-soybean plots and sometimes affected yield. The largest effect was at Carman in 2013. Volunteer soybeans contributed an average 1715 kg ha^{-1} to yield at Carman in 2013. Soybean-soybean plots yielded 4448 kg ha^{-1} including volunteers, but only 2733 kg ha^{-1} without volunteers. If volunteers had not been present, however, this yield would have been greater as there would be less competition for light and nutrients. When volunteer yield was not included the soybean-soybean sequence yielded significantly lower than the other three sequences at Carman in 2013. At Kelburn in 2013, volunteer soybean contributed an average 320 kg ha^{-1} to yield. Soybean-soybean plots at Kelburn in 2013 yielded 2832 kg ha^{-1} including volunteers, and 2512 kg ha^{-1} without volunteers. Volunteer yield did not significantly affect soybean-soybean sequence yield compared to other crop sequences at Kelburn in 2013. The contribution of volunteers to soybean yield were included in all final yield calculations as volunteer soybeans would impact the yield of non-volunteer soybeans through increased competition for nutrients. A small number of volunteer soybeans emerged at Kelburn in 2014, which were removed by hand. There were no volunteers detected at Carman and Portage in 2014.

4.2 Colonization of Soybean Roots by Arbuscular Mycorrhizal Fungi

4.2.1 Effect of Crop Sequence on Arbuscular Mycorrhizal Fungi Colonization

The results of this experiment showed that crop sequence had a significant impact on arbuscular mycorrhizal colonization of the soybean roots (Table 4.3). The inclusion of a non-mycorrhizal crop significantly affected mycorrhizal colonization after one year (Table 4.3). AMF colonization was quantified in three measurements: percent hyphae, percent arbuscules and percent total colonization (hyphae, arbuscules and vesicles, respectively). These measurements are defined as the number of root gridline intersects where a mycorrhizal structure is present, divided by the total number of root gridline intersects. Site years were combined for analysis of total colonization as there was not a significant interaction between treatment and site year in terms of total percent colonization (Table 4.3). There was, however, an interaction between treatment and site year for percent hyphae and percent arbuscules, so these variables were analysed by site year (Table 4.4).

Table 4.3. Effect of preceding crop (Crop) and site year (SiteYear) on mycorrhizal colonization of soybean roots at the V3 stage by percent hyphae, percent arbuscules and total percent colonization pooled across five site years.

Factor	Treatment	% Hyphae†	% Arbuscules	Total % Colonization‡
Crop	Canola	29.7 **c**	23.5	41.8 **b**
	Corn	43.9 **a**	22.9	53.5 **a**
	Soybean	44.0 **a**	24.0	54.0 **a**
	Wheat	33.8 **b**	22.6	45.1 **b**
SiteYear	Carman 2013	43.2 **a**	29.2 **a**	57.7 **a**
	Carman 2014	34.7 **b**	25.7 **ab**	45.5 **c**
	Kelburn 2013	37.1 **ab**	24.3 **ab**	54.1 **ab**
	Kelburn 2014	31.3 **b**	15.3 **c**	36.2 **d**
	Portage 2014	43.0 **a**	21.7 **bc**	49.4 **bc**
ANOVA			P > F	
Crop		<.0001	0.7767	<.0001
SiteYear		0.0051	0.0064	<.0001
Crop*SiteYear		0.0006	0.0229	0.0743

†Percent hyphae defined as percentage of times that a root contained hyphae at a root gridline intersection

‡ Total percent colonization defined as hyphae, arbuscules and vesicles combined.

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Table 4.4. Effect of preceding crop and site year on mycorrhizal colonization in soybean roots at the V3 stage by percent hyphae and percent arbuscules at Carman (2013, 2014), Kelburn (2013,2014) and Portage (2014).

	Carman		Kelburn		Portage
Treatment	2013	2014	2013	2014	2014
	----------Percent Hyphae[†]----------				
Canola	34.9 **c**	29.9 **b**	26.6 **b**	18.6 **c**	38.8
Corn	44.8 **ab**	37.5 **ab**	44.8 **a**	46.0 **a**	46.6
Soybean	50.1 **a**	41.2 **a**	50.5 **a**	32.1 **b**	46.0
Wheat	43.2 **b**	30.1 **b**	26.4 **b**	28.7 **b**	40.6
	----------Percent Arbuscules----------				
Canola	30.3	23.6 **ab**	29.9 **a**	13.9	20.0
Corn	28.2	22.9 **b**	22.8 **bc**	18.0	22.4
Soybean	32.2	30.0 **a**	18.7 **c**	16.1	22.8
Wheat	26.2	26.3 **ab**	25.7 **ab**	13.2	21.5

[†]Percent hyphae defined as percentage of times that a root contained hyphae at a root gridline intersection

Means within a column followed by a different letter are statistically significant at $P<0.05$ according to Fisher's Protected LSD.

The soybean-soybean and corn-soybean rotations resulted in greater total percent mycorrhizal colonization than wheat-soybean and canola-soybean (Table 4.3). The canola-soybean sequence resulted in significantly less total percent AMF colonization than the soybean-soybean and corn-soybean sequences, but equivalent to the wheat-soybean sequence when averaged over all site years. Soybean-soybean often resulted in significantly higher percent hyphae than wheat-soybean and canola-soybean (Table 4.4). Soybean-soybean had significantly higher percent hyphae than canola-soybean at every site year except Portage in 2014. Soybean-soybean also had significantly higher percent hyphae than wheat-soybean at Kelburn in 2013, and at Carman in 2013 and 2014. Corn-soybean had significantly higher percent hyphae than canola-soybean at Carman in 2013, and Kelburn in 2013 and 2014 (Table 4.4). Corn-soybean also had higher percent hyphae

than wheat-soybean at Kelburn in 2013 and 2014. Canola-soybean often had significantly lower percent hyphae than all other rotations (Table 4.4). Canola-soybean had less percent hyphae than all other rotations at Carman in 2013 and Kelburn in 2014. This is likely due to the fact that a non-mycorrhizal crop will reduce the quantity of fungal inoculum in the soil, which includes both spores and hyphae. There were no differences across crop sequence treatments in percent arbuscules when site years were combined.

There were few significant differences between treatments in terms of percent arbuscules (Table 4.4). The soybean-soybean sequence and the corn-soybean sequence had significantly less percent arbuscules than canola-soybean at Kelburn in 2013. The corn-soybean sequence had significantly less percent arbuscules than the soybean-soybean sequence at Carman in 2014. Arbuscules are the main site of nutrient exchange between plant and AMF, and because of this important function some studies have only measured percent arbuscules (Smith and Gianinazzi-Pearson, 1990; Ezawa et al., 2002). Arbuscules can be more difficult to quantify, however, as smaller arbuscules can be difficult to detect (McGonigle et al., 1990), arbuscules can be easily confused with other structures in the root system (Dodd and Jeffries, 1986), and arbuscles are very short-lived (Treseder, 2013). These factors may have made the quantification of arbuscules in this experiment more difficult and less accurate.

Previous research has shown that non-mycorrhizal crops grown before soybean reduce mycorrhizal colonization. Chen et al. (2013) found that total AMF colonization in soybean following canola and sugar beet (both are non-mycorrhizal crops) was 14 to 18 percent lower than soybean following mycorrhizal crops (corn, wheat, Illinois

bundleflower, soybean, alfalfa, sunn hemp and sunflower). An et al. (1993) found that soybean grown on four different mycorrhizal crops (corn, fescue, sorghum and soybean) all had similar levels of colonization, indicating that crop rotation does not have a strong effect on mycorrhizal colonization if the previous crop was mycorrhizal.

Mycorrhizal crops other than soybean also see similar declines when grown on a non-mycorrhizal crop stubble (Harinikumar and Bagyaraj, 1988; Douds et al., 1997; Gavito and Miller, 1998; Karasawa et al., 2002; McGonigle, 2009). Harinikumar and Bagyaraj (1988) found AMF colonization in cowpea decreased 13 percent after one year of a non-mycorrhizal crop. The same study found that leaving land fallow resulted in an even greater decline in AMF colonization, however, decreasing AMF colonization by 40 percent in the following crop.

Research conducted in Manitoba has shown that crop rotation can affect the degree of mycorrhizal colonization. Welsh (2007) found that a forage-grain rotation consisting of flax, alfalfa and wheat had higher AMF colonization than a grain-only rotation consisting of flax, oat, faba bean and wheat. This is consistent with the results of this experiment, which show that inclusion of a non-mycorrhizal or weakly mycorrhizal crop can significantly reduce mycorrhizal colonization.

The use of tillage to manage crop residues may have influenced the results of this experiment. The tillage implements, timing, and number of tillage passes was selected to represent current management used on commercial farms in Manitoba and was not the same for each treatment crop in the experiment. All plots were cultivated with shanks once in the fall after harvest of the treatment crop and once in the spring before seeding the soybean test crop. The first year corn treatment plots were also disced once in the fall

after corn harvest and twice in the spring before planting the soybean test crop. All other treatments did not receive this additional tillage amendment. Tillage has been shown to decrease AMF colonization (Abbott and Robson, 1991). Despite this, the corn-soybean sequence had high levels of mycorrhizal colonization. Results from this experiment suggest that crop rotation may be just as influential as tillage in terms of AMF colonization.

4.2.2 Soil Test Phosphorus Levels

Soil test P levels before the soybean test crop varied significantly by site year and crop sequence treatment (Table 4.5, Table 4.6). Soil samples were taken in early May at a depth of 0-15 cm. Although most site years had soil P levels between 7 and 15 parts per million (ppm), soil P was considerably higher at Kelburn in 2014, ranging from 26 to 35 ppm (Table 4.6). Results were analyzed by site year as there was a significant treatment by site year interaction (Table 4.5).

Table 4.5. Effect of preceding crop (Crop) and site year (SiteYear) on soil test phosphorus (Olsen P) at 0-15 cm prior to seeding soybean test crop plots.

Factor	Treatment	Soil Test P (ppm)
Crop	Canola	14.4
	Corn	14.9
	Soybean	12.8
	Wheat	15.2
SiteYear	Carman 2013	10.3 **bc**
	Carman 2014	9. 3 **c**
	Kelburn 2013	12.9 **b**
	Kelburn 2014	29.3 **a**
	Portage 2014	9.8 **bc**
ANOVA		P > F
Crop		0.4331
SiteYear		<.0001
Crop*SiteYear		0.0067

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Table 4.6. Soil test phosphorus (Olsen P) at 0-15 cm prior to seeding soybean test crop based on preceding crop (Crop) at Carman (2013, 2014), Kelburn (2013, 2014) and Portage (2014).

	Carman		Kelburn		Portage
Treatment	2013	2014	2013	2014	2014
	----------Soil P ppm----------				
Canola	11.5 **ab**	12.0 **a**	14.0	26.0	8.5
Corn	12.0 **a**	9.8 **b**	14.3	27.0	11.5
Soybean	8.3 **c**	8.3 **bc**	10.0	29.5	7.8
Wheat	9.3 **bc**	7.3 **c**	13.3	34.8	11.3
ANOVA			P > F		
Crop	0.0271	0.0014	0.1587	0.1465	0.095

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Differences in soil P among treatments varied by site year. At Carman in 2013, soil P following corn was significantly higher than soil P following wheat or soybean, while soil P following soybean was significantly lower than soil P following canola or

corn. At Carman in 2014, soil P after canola was higher than all other sequences, while soil P following wheat was lower than soil P following corn or canola. At Kelburn in 2013, soil P following corn was higher than soil P following soybean. At Kelburn in 2014, soil P following wheat was higher than soil P following canola. At Portage in 2014, soil P following corn was higher than soil P following soybean. Soil P was lowest following soybean at three of five site years (Table 4.6). There was no relationship between soil P and yield (P=0.2967), although soil P did negatively affect mycorrhizal colonization (Figure 4.1).

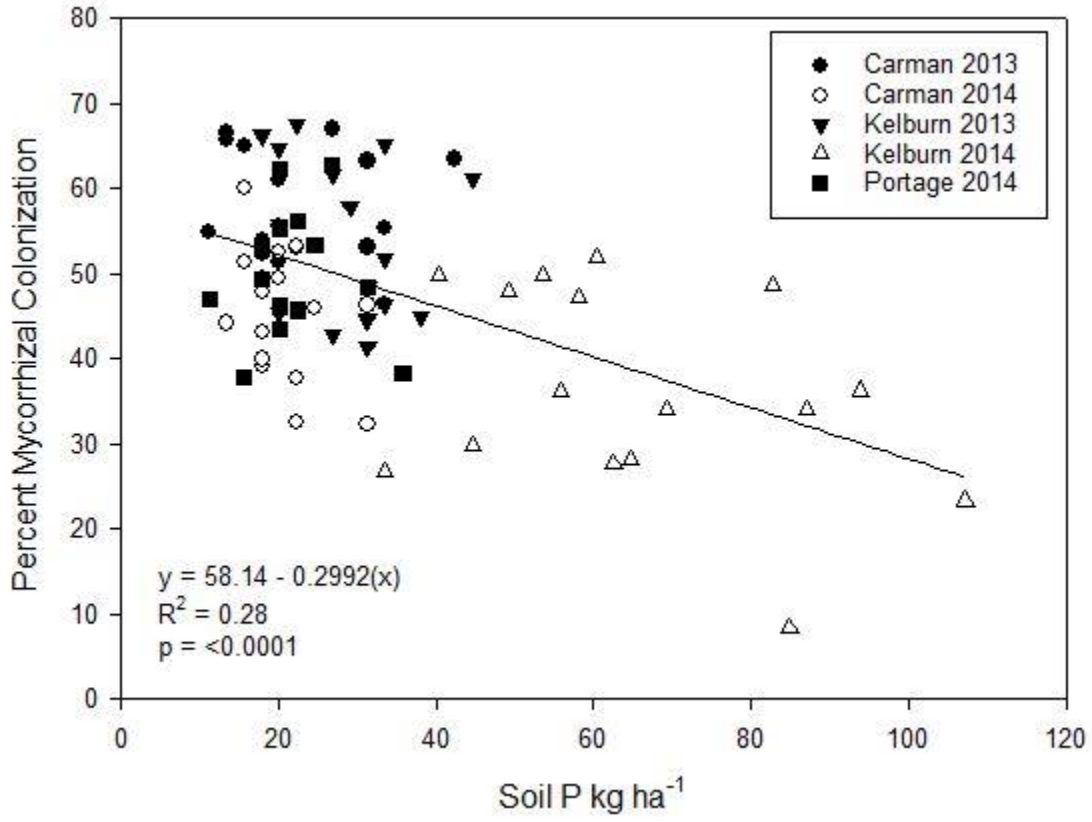

Figure 4.1. Effect of spring soil test phosphorus levels on mycorrhizal colonization of soybean roots at V3 stage. Soil sampling was performed at a depth of 0-15 cm in early May before seeding.

Phosphorus fertilizer was applied to all first year treatment crops except soybean, as this reflects current management practices for soybean in Manitoba (Table 3.5). Rates were based on soil test recommendations and varied by site year. Wheat received less P than canola and corn at Carman in 2013 and Portage in 2013. Soil P following wheat was lower than soil P following corn at Carman in 2013, but was not different from any other crop sequence at Portage in 2014. The lack of a P amendment may have contributed to soybean having the lowest soil P levels at three site years. The higher concentration of soil P at Kelburn, particularly in 2014, may be due to the high clay content of soil in that area. Clay has a higher capacity for adsorbing ions in the soil, and as a result often has higher soil P levels (Malavolta, 1980).

Previous research has shown that soybean takes up and removes significant amounts of phosphorus. Provincial soil fertility guidelines estimate that a 2350 kg ha^{-1} soybean crop removes 14 kg P ha^{-1}. Bardella (2016) found that a 2700 kg ha^{-1} soybean crop in Manitoba removed 17 kg P ha^{-1} annually. Canola and corn remove even more P (26 and 21 kg P ha^{-1}). Wheat removes the least P (11 kg P ha^{-1}). Residual soil P in the canola-soybean and corn-soybean sequences, however, were not lower than the other crop treatments. Soybean following wheat had higher soil P than soybean following canola at Kelburn in 2014, but lower soil P than canola-soybean and corn-soybean at Carman in 2014. This discrepancy in P removal rates and soil P levels indicates that soil P is affected by long term management practices as well as previous crop removal.

Soil test P levels before the soybean test crop had a significant effect on mycorrhizal colonization (Figure 4.1). Mycorrhizal colonization was negatively correlated with increasing spring soil test P levels. There was a weak relationship

between P fertilizer rates for the preceding treatment crops and mycorrhizal colonization in the soybean test crop (P=0.0548, R^2=0.05)(Figure 7.1). Previous research has found that high soil P and application of fertilizer P reduces AMF colonization (Abbott et al., 1984; Thomson et al., 1986; Harinikumar and Bagyaraj, 1989; Miranda and Harris, 1994; Tang et al., 2001). Colonization can be negatively affected even at low levels of fertilizer P application (Clapperton et al., 1997). Some studies, however, have found little to no effect of soil P or fertilizer P application on mycorrhizal colonization (Ellis et al., 1992; Eriksson, 2001). A meta-analysis of twenty studies of various crops from around the world found that a P amendment reduced mycorrhizal colonization by an average of 32 percent (Treseder, 2004). All studies in this meta-analysis looked at mycorrhizal response to manipulation of soil P levels over more than two months. The response ratio of mycorrhizal colonization to P was very consistent across all studies in this meta-analysis, regardless of biome, type of fertilizer applied, rate of fertilizer applied or duration of fertilization. This indicates that AMF colonization is affected by soil test P levels as opposed to soil P fertilization itself. In Manitoba, Welsh (2007) found that flax had significantly higher AMF colonization in soils with low soil test P levels than flax grown in soils with higher levels of soil phosphorus. This is consistent with the results of this study, which showed that mycorrhizal colonization responded more to residual soil P levels than P fertilization.

4.2.3 Phosphorus Concentration of Soybean Plants

Phosphorus concentration was measured for the 2014 site years from samples taken at the V3 and R5 stage. Site years were combined at the V3 stage as there was not a significant interaction between treatment and site year (Table 4.7).

Table 4.7. Phosphorus concentration of V3 soybean shoots as affected by preceding crop (Crop) and site year (SiteYear).

Factor	Treatment	mg P / kg dry plant biomass
Crop	Canola	2300 **b**
	Corn	3000 **a**
	Soybean	3000 **a**
	Wheat	2900 **a**
SiteYear	Carman 2014	2300
	Kelburn 2014	2600
	Portage 2014	2500
ANOVA		P > F
Crop		0.0007
SiteYear		0.0009
Crop*SiteYear		0.2351

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

There were significant differences in P concentration based on crop sequence treatment at the V3 stage (Table 4.7). The canola-soybean crop sequence had significantly lower plant P concentration than all other crop sequences at the V3 stage. This could be due to the large amount of P that canola removes from the soil, although corn removes similarly high levels of P and soil P following canola was not lower than other crop sequences. Increased P uptake via increased AMF colonization could also have played a role in plant P concentrations. Soybean following canola had significantly lower mycorrhizal colonization than soybean following corn and soybean, and this could have affected the plant's P uptake ability, especially early in the growing season. There were no significant differences in plant P concentration between the other three crop sequences.

There were fewer significant differences in P concentration at the R5 stage of development (Table 4.8, Table 4.9). Results were analysed by site year as there was a

significant site year by treatment interaction effect. On average soybean shoot P concentration was greater at the V3 stage of development than the R5 stage of development. Average soybean shoot P concentration (across all site years and treatments) at the V3 stage was 2800 mg P per kg plant biomass on a dry matter basis, while at the R5 stage it was 2200 mg P per kg plant biomass. At Kelburn in 2014, the corn-soybean sequence had higher P concentration than all other crop sequences, while the canola-soybean rotation had lower P concentration than all other sequences. There were no treatment differences at Carman or Portage in 2014 at the R5 stage (Table 4.9). The effect of decreased AMF colonization in soybean following canola may have affected plant P concentration in soybean following canola at Kelburn in 2014, but the trend did not appear to extend to the other two site years. This suggests that later in development the advantage in plant P concentration provided by increased AMF colonization may decrease.

Table 4.8. Analysis of variance (ANOVA) of effect of preceding crop (Crop) and site year (SiteYear) on phosphorus concentration of R5 soybean shoots.

ANOVA	P > F
SiteYear	0.0013
Crop	0.2303
Crop*SiteYear	0.0012

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Table 4.9. Phosphorus concentration of R5 soybean plants as affected by preceding crop (Crop) harvested at three site years in 2014.

	Carman	Kelburn	Portage
Treatment	2014	2014	2014
	----------mg P / kg dry plant biomass----------		
Canola	2300	1400 **c**	2700
Corn	2300	2300 **a**	2400
Soybean	2200	1900 **b**	2300
Wheat	2200	1800 **b**	2400
ANOVA		P > F	
Crop	0.8446	0.0011	0.1629

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

The relationship between mycorrhizal colonization and plant P concentration depended on plant development stage. There was not a significant relationship between mycorrhizal colonization and plant P concentration at the V3 stage (P=0.5370)(R^2=0.008)(Figure 7.2). There was, however, a significant relationship between mycorrhizal colonization and plant P concentration at the R5 stage of development. Mycorrhizal colonization had a positive exponential relationship with plant P concentration at the R5 stage of development (Figure 4.2).

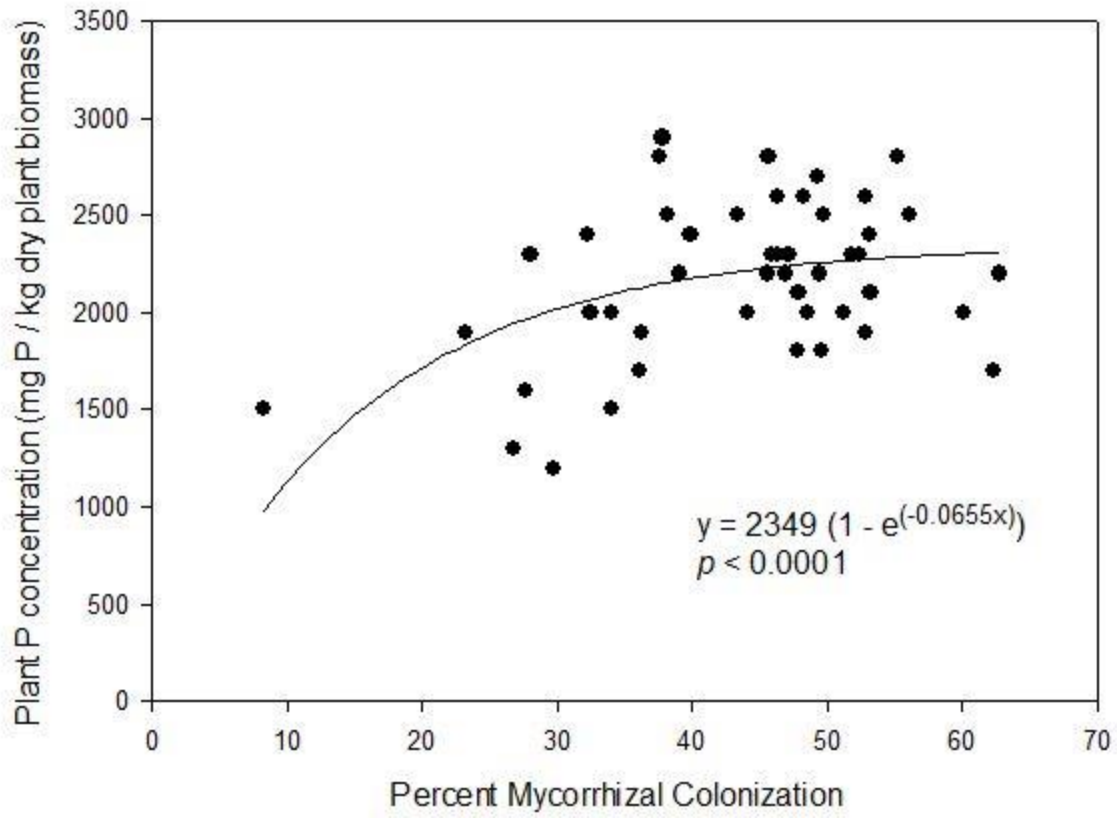

Figure 4.2. Effect of mycorrhizal colonization on soybean shoot phosphorus concentration at R5 stage (mg phosphorus per kg dry plant biomass).

Mycorrhizae have been shown to increase seed P concentration. Bethlenfalvay et al. (1997) compared soybean grown in pot cultures that were inoculated with AMF against soybean that were not inoculated. Some of the non-inoculated soybean received a P amendment. They found that inoculated soybean had significantly higher seed P concentration than those that were not inoculated, even compared to the ones that had a received a P amendment. The non-inoculated soybean that received a P amendment, however, yielded significantly higher than the inoculated soybean, and seed P content was not correlated with increased yield or seed protein content. This indicates that while AMF contribute to seed P concentration, this may not translate into an increase in seed yield or seed protein content.

4.2.4 Relationship between Mycorrhizal Colonization and Soybean Yield

Mycorrhizal colonization was slightly correlated with yield. Increasing mycorrhizal colonization was correlated with a slight increase in yield when averaged across all five site years (Figure 4.3). Correlation analysis of yield and mycorrhizal colonization produced an R value of 0.263. (Figure 4.3).

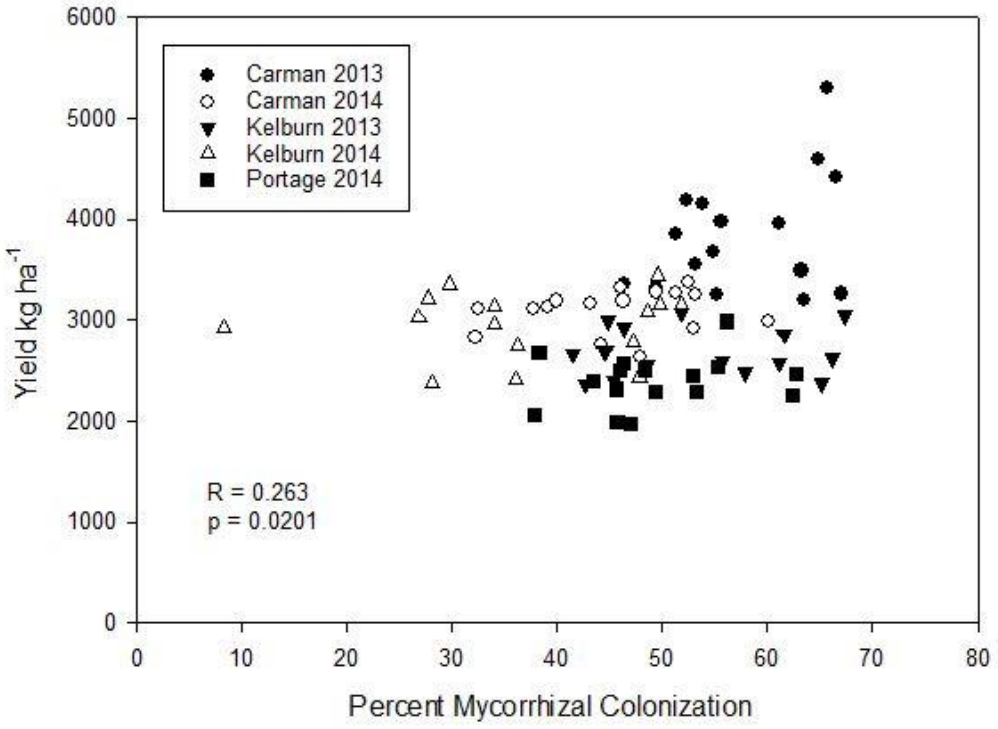

Figure 4.3. Correlation between mycorrhizal colonization and soybean yield pooled across five site years.

Previous research shows AMF have the largest impact on yield when residual P levels are low. In a pot culture experiment using a sandy-loam soil, Bethlenfalvay et al. (1997) compared seed yield of soybean plants that were inoculated with three strains of AMF to soybean plants that were not inoculated but received a P amendment of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, 1 g kg^{-1} soil] or no P amendment. Olsen P of the soil before addition of the P amendment was 10 parts per million. They found that soybean

plants inoculated with AMF had 65 percent greater seed yield than plants that had not been inoculated and had not received a P amendment. Conversely, they found that plants inoculated with AMF yielded 42 percent less than plants that were not inoculated but received a P amendment. Another pot culture experiment found that inoculation of soybean roots with AMF increased soybean yield by 7 to 45 percent, with the increase in yield largely dependent upon soil type and mycorrhizal strain (Young et al., 1986). Leaf senescence began sooner in plants that had not been inoculated, potentially reducing photosynthate supply to seeds. All site years in this experiment had residual soil P levels higher than the 10 ppm baseline used in Bethlenfalvay et al. (1997). The effect of mycorrhizal colonization on yield may have been larger if soil P levels had been lower.

4.3 Biological Nitrogen Fixation in Soybean Roots by *Bradyrhizobium*

4.3.1 Effect of Crop Sequence on Biological Nitrogen Fixation in Soybean Roots

Crop sequence had a significant effect on %Ndfa (Table 4.10,Table 4.11). Results were analyzed by site year as there was a significant site year by treatment interaction.

Table 4.10. Analysis of variance (ANOVA) of effect of preceding crop (Crop) and site year (SiteYear) on %Ndfa in soybean at R5 and R6 stage.

ANOVA	P > F
Crop	<.0001
SiteYear	0.0038
Crop*SiteYear	<.0001

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Table 4.11. Percent of nitrogen in soybean plant fixed biologically at the R5 growth stage based on preceding crop (Crop) at Carman (2013, 2014), Kelburn (2013, 2014) and Portage (2014).

	Carman			
Treatment	2013		2014	
	%Ndfa	Range†	% Ndfa	Range
Canola	67.6 **c**	60.1 - 77.2	39.0 **c**	34.6 - 44.5
Corn	73.4 **bc**	66.0 - 82.8	71.0 **a**	62.9 - 82.4
Soybean	80.3 **a**	71.7 - 91.2	53.7 **b**	48.1 - 61.9
Wheat	79.3 **ab**	71.0 - 89.8	62.2 **ab**	54.9 - 71.8
ANOVA	P > F			
Crop	0.0035		<.0001	
	Kelburn			
Treatment	2013		2014	
	% Ndfa	Range	% Ndfa	Range
Canola	60.9 **c**	55.4 - 67.4	58.7 **b**	53.6 - 64.9
Corn	73.1 **b**	67.8 - 79.3	73.6 **a**	68.7 - 81.8
Soybean	84.3 **a**	78.1 - 92.7	56.0 **b**	51.5 - 61.5
Wheat	64.7 **c**	59.3 - 71.4	56.2 **b**	51.7 - 61.6
ANOVA	P > F			
Crop	<.0001		<.0001	
	Portage			
Treatment	2014			
	% Ndfa	Range		
Canola	55.3 **b**	50.3 - 61.3		
Corn	77.6 **a**	70.5 - 86.1		
Soybean	45.9 **c**	41.4 - 51.5		
Wheat	59.1 **b**	53.5 - 66.1		
ANOVA	P > F			
Crop	<.0001			

†Range refers to potential range of %Ndfa as measured by isotopic fractionation with a mass spectrometer using the ^{15}N natural abundance method. The low and high end of the reported range is calculated by highest and lowest B value reported for temperate environments in Unkovich et al. (2008).
Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Corn-soybean and soybean-soybean rotations often had higher %Ndfa than canola-soybean and wheat-soybean rotations, although treatment effects varied by site

year. At Carman in 2013, soybean-soybean and wheat-soybean had higher %Ndfa than canola-soybean. At Carman in 2014, corn-soybean and wheat-soybean had higher %Ndfa than soybean-soybean, while canola-soybean had lower %Ndfa than all other crop sequences. At Kelburn in 2013, soybean-soybean had higher %Ndfa than all other sequences, while corn-soybean had higher %Ndfa than canola-soybean and wheat-soybean. At Kelburn in 2014, the corn-soybean sequence had significantly higher %Ndfa than all other sequences. At Portage in 2014, corn-soybean had higher %Ndfa than all other sequences, while soybean-soybean had lower %Ndfa than all other sequences (Table 4.11). When site years were combined, soybean-corn had higher %Ndfa than all other treatments, while soybean-canola had lower %Ndfa than all other treatments.

Total nitrogen fixed biologically was calculated using soybean test crop biomass samples in 2013. There was no biomass data available in 2014. There were no significant differences in Kg N ha^{-1} fixed based on preceding crop. Site years were combined as there was not a significant site year interaction effect.

Table 4.12. Effect of preceding crop (Crop) and site year (SiteYear) on total nitrogen fixed biologically by soybean at R5 and R6 stage.

Factor	Treatment	Total N Fixed at R5 Stage (Kg $ha^{-1)}$	Total N Fixed at R6 Stage (Kg $ha^{-1)}$
Crop	Canola	103.1	103.8
	Corn	116.5	134.2
	Soybean	138.6	119.4
	Wheat	113.4	119.5
SiteYear	Carman 2013	141.4 **a**	116.3
	Kelburn 2013	94.3 **b**	122.2
	ANOVA	P>F	
	Crop	0.1297	0.3167
	SiteYear	0.0001	0.6033
	Crop*SiteYear	0.9227	0.5308

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

There was a significant negative relationship between %Ndfa and test soil N after the treatment crop when data was combined across site years. Regression analysis indicates a linear relationship between %Ndfa and soil N, with %Ndfa decreasing with increasing soil N (Figure 4.4). There was no significant relationship between C:N ratio of previous crops and %Ndfa (P=0.1120)(Figure 7.3). There was no relationship between soybean yield and %Ndfa (Figure 7.4).

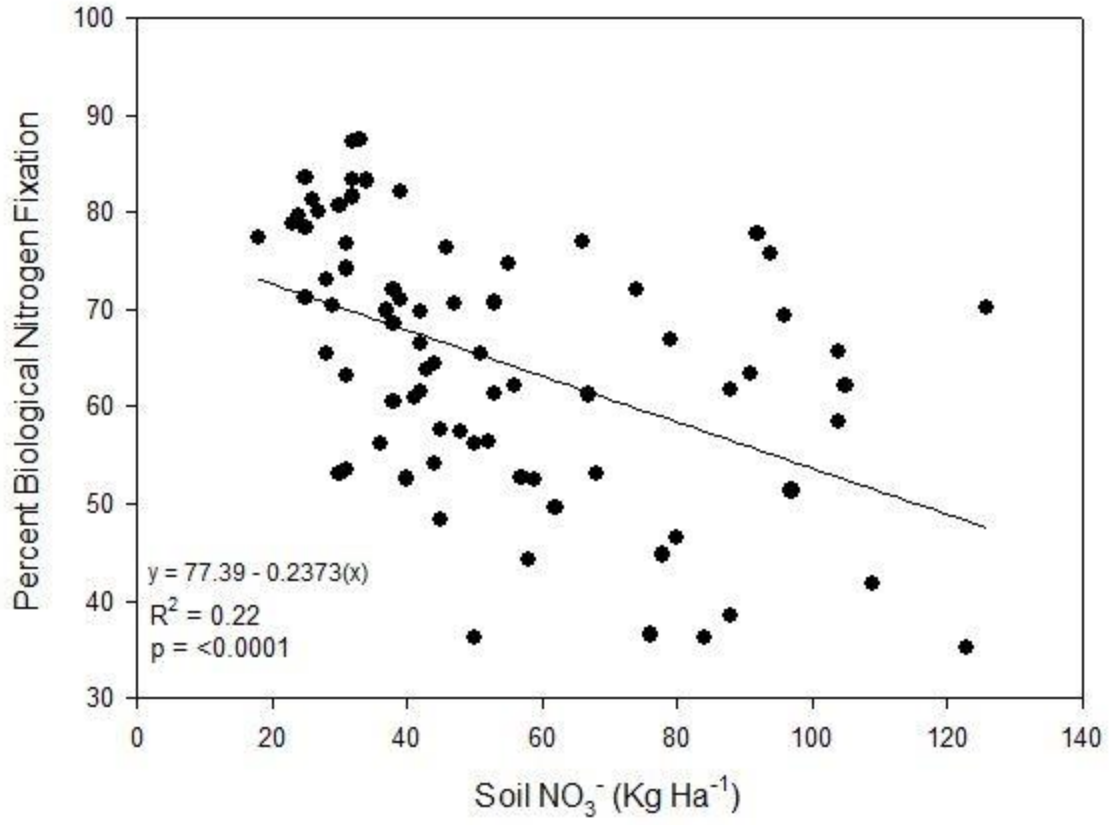

Figure 4.4. Effect of soil test nitrogen (NO_3^-) on biological nitrogen fixation in soybean pooled across five site years.

Soil N was often higher following the canola treatment crop. Corn and soybean often resulted in lower levels of spring soil test N, resulting in the plant becoming more dependent on BNF to meet its N requirements. The lack of a fertilizer amendment on the soybean reference crops likely decreased soil N relative to other first year crops that did receive an N amendment.

Numerous studies have found that increasing presence of mineral nitrogen results in a decrease in BNF (Bergersen et al., 1989; Brockwell et al., 1989; Peoples and Herridge, 1990; Fujikake et al., 2002; Saito et al., 2014). Biological nitrogen fixation is

an energy intensive process. When residual soil nitrogen is available to the plant, it is more efficient for the plant to take up nitrogen rather than fix it from the atmosphere. Rhizobia decrease nitrogen fixing activity in the presence of nitrates near root nodules (Vessey and Waterer, 1992). The inhibition of nitrogenase and legheamoglobin (enzymes responsible for the reduction of N_2 to NH_3) by soil nitrates inhibits the formation of nodules (Zahran, 1999). Nitrate inhibition also results in decreased nodule numbers, nodule mass, BNF activity and acceleration of nodule senescence (Saito et al., 2014).

Crop sequence can also affect %Ndfa through the C:N ratio of the crop stubble and the amount of residual nitrogen left behind by each crop. A higher C:N ratio results in more soil N being immobilized by bacteria and thus unavailable to plants (Brady and Weil, 2008). This results in less available soil nitrogen. Carbon to nitrogen ratios were significantly correlated with %Ndfa at Carman and Portage in 2014 (Figure 4.5).

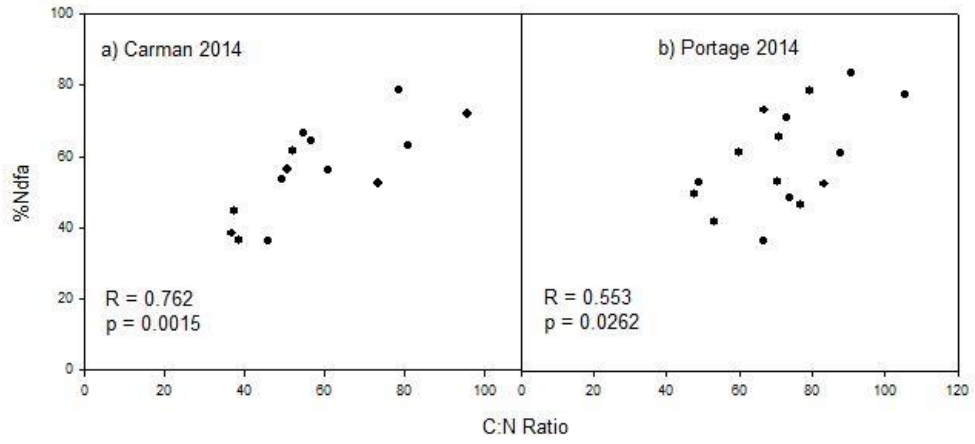

Figure 4.5. Correlation between carbon to nitrogen (C:N) ratios of preceding crops and biological nitrogen fixation in soybean plants at Carman and Portage in 2014.

Biological nitrogen fixation increased as C:N ratios increased at both site years. There was a significant relationship between C:N ratio and soil N at Carman and Portage in 2014, but not at the other three site years and not when all five site years were combined. Carbon to nitrogen ratios affected %Ndfa at two site years, but the trend was not consistent throughout all experiments in the study.

4.3.2 Soil Test Nitrogen Levels

Soil test N after the treatment crops varied between site years and crop sequence treatments (Table 4.13, Table 4.14). Results were analyzed by site year as there was a significant site year by treatment interaction effect. Soil sampling for nitrate levels was conducted in early May at all sites, at a depth of 0-60 cm in two increments (0-15 cm and 15-60 cm).

Table 4.13. Effect of preceding crop (Crop) and site year (SiteYear) on spring soil test nitrate levels (NO_3^-) at 0-60 cm in the subsequent soybean crop prior to planting in May.

ANOVA	P > F
Crop	<.0001
SiteYear	<.0001
Crop*SiteYear	<.0001

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Table 4.14. Effect of preceding crop (Crop) on spring soil test nitrate (NO_3^-) levels at 0-60 cm in the subsequent soybean crop prior to planting in May at Carman (2013, 2014), Kelburn (2013, 2014) and Portage (2014).

	Carman		Kelburn		Portage
Treatment	2013	2014	2013	2014	2014
	----------Soil N kg ha^{-1}----------				
Canola	43	73 **a**	105 **a**	46	67 **a**
Corn	33	30 **c**	85 **b**	55	29 **b**
Soybean	28	38 **bc**	35 **c**	41	67 **a**
Wheat	30	45 **b**	98 **ab**	68	54 **a**
P > F	0.1129	0.0004	<.0001	0.231	0.0077

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Spring soil test N varied from 105 kg ha^{-1} in the canola-soybean rotation at Kelburn in 2013 to 28 kg ha^{-1} in the soybean-soybean rotation at Carman in 2013 (Table 4.14). The canola-soybean rotation often resulted in higher spring soil test N compared to other crop sequences. The corn-soybean sequence often resulted in lower spring soil test N than other rotations. At Carman in 2014, canola-soybean had higher spring soil test N than all other crop sequences, while corn-soybean had lower spring soil test N than canola-soybean and wheat-soybean. At Kelburn in 2013, canola-soybean had higher spring soil test N than all other crop sequences, while soybean-soybean had lower spring soil test N than all other sequences. There were no significant differences in spring soil test N between treatments at Carman or Kelburn in 2014. At Portage in 2014, corn-soybean had significantly lower spring soil test N than all other crop sequences.

Fertilization rates of the preceding crop affected spring soil test N levels in the subsequent soybean crop (P = 0.0397)(Figure 4.6). Corn and canola received the highest levels of N fertilizer based on soil tests and target yield goals, with rates varying from 50 to 100 kg N ha^{-1} (Table 3.5). Wheat usually received about half as much nitrogen as corn

and canola, with rates ranging from 28 to 67 kg N ha^{-1}. The soybean reference crops did not receive any nitrogen fertilizer. These fertilization patterns were reflected in soil test N levels the next year. Although fertilization rates in the preceding crops affected spring soil test N levels, the low R^2 value (0.05) indicates that other factors had a strong influence on spring soil test N levels and fertilization was not the most significant factor.

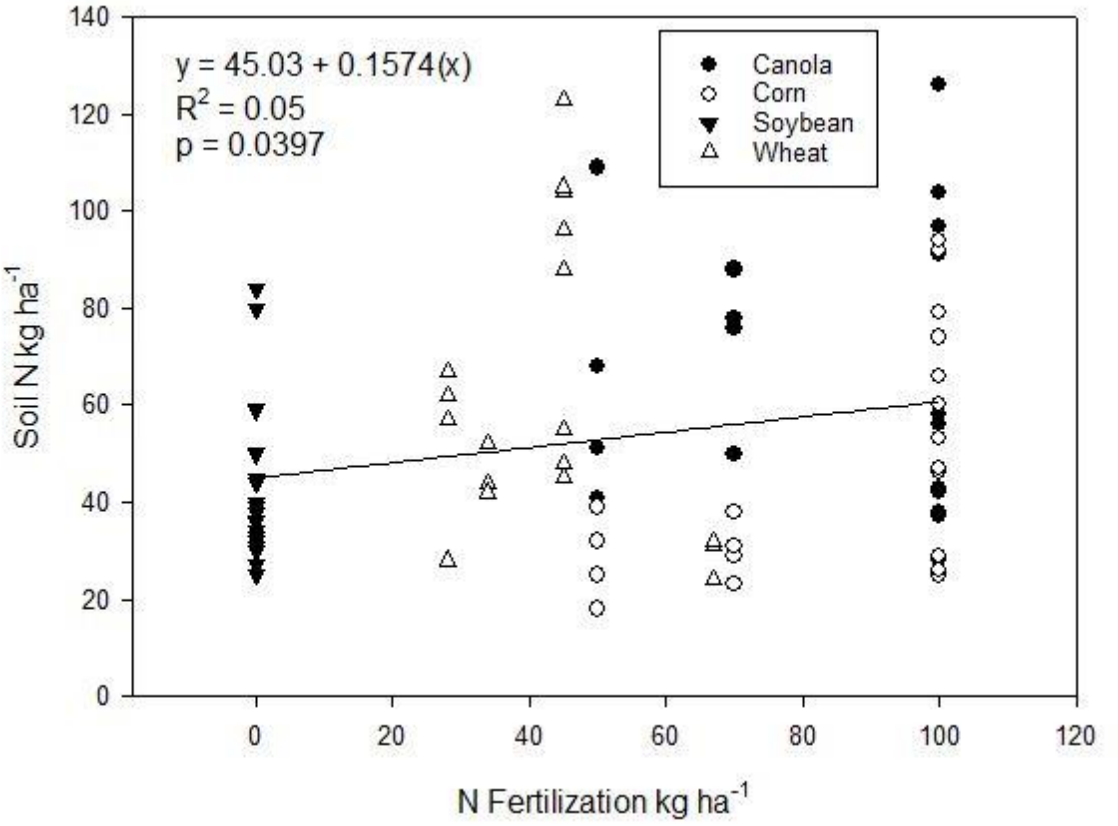

Figure 4.6. Effect of NH_4^+ and NO_3^- fertilization rate of preceding crop on spring soil test N levels (NO_3^-) at a depth of 0-60 cm in soybean test crop prior to planting in May.

Despite receiving the same amount of N fertilizer, canola-soybean plots had significantly higher soil N than corn at three of five site years. This is likely due to the higher C:N ratio of corn compared to other crops, and the subsequent nitrogen immobilization that occurs because of it. Canola-soybean plots had the highest levels of soil N at four of five site years, and never had significantly lower soil N levels than any

other crop sequence. Corn-soybean plots had the lowest levels of soil N at two site years. The soybean-soybean plots had the lowest levels of soil N at three site years.

Nitrogen removal by each crop affected the level of soil N left behind. In Manitoba, N removal rates are typically greatest for soybean, followed by corn, canola, and wheat (MAFRD, 2007). Soybean typically removes 179-224 kg ha^{-1} every year, corn removes 154-188 kg N ha^{-1}, canola removes 112-138 kg N ha^{-1}, and spring wheat removes 85-104 kg N ha^{-1} (MAFRD, 2007). The high amount of N removal by soybean, combined with the lack of fertilizer amendment, explain why soybean-soybean plots had the lowest residual N at three site years. The higher residual N levels of canola-soybean plots reflects the higher removal rate of corn, despite the fact that both received the same fertilizer amendment.

Carbon to nitrogen ratios of crop residue affect residual N levels. Crops with higher C:N ratios tend to immobilize more N in the soil, as the microbes breaking down the residues cannot get enough N from the residue itself (Brady and Weil, 2008). When site years were combined there was no significant relationship between C:N ratios and soil nitrogen. There was a significant linear relationship between C:N ratios and soil N at Carman in 2014 and a significant linear relationship at Portage in 2014 (Figure 4.7). This may have been due to the higher levels of biomass produced at Portage and Carman in 2014 compared to Kelburn in 2014 (Table 7.2, Table 7.3). Increased levels of biomass may have resulted in increased microbial populations breaking down crop residue and immobilizing nitrogen, creating a stronger relationship between C:N ratio and spring soil test nitrogen. The higher levels of biomass at Portage and Carman, however, were not

statistically greater compared to Kelburn (P=0.44). First year treatment crop biomass data was not available for trials conducted in 2012-2013.

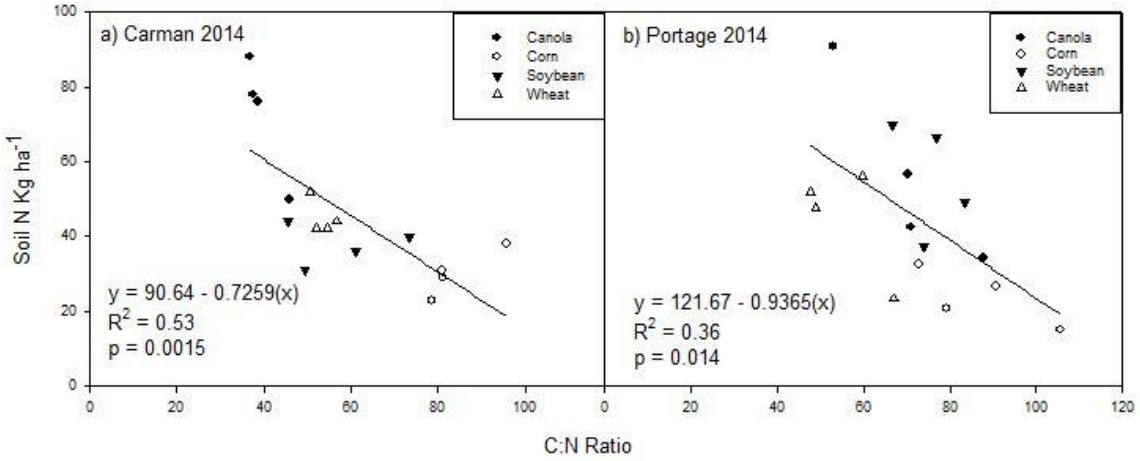

Figure 4.7. Effect of carbon to nitrogen (C:N) ratios of preceding crops on soil test N (NO_3^-) at Carman and Portage in 2014. Soil sampling was performed at a depth of 0-60 cm in early May before seeding.

Most research suggests the nitrogen benefit of soybean as a result of BNF is slightly negative to neutral (Salvagiotti et al., 2008). Although some studies have found a net nitrogen benefit, a meta-analysis of 637 studies found that the average N balance in the soil after a soybean crop was -4 kg N ha^{-1} (Salvagiotti et al., 2008). Although BNF contributes 50-60% of the plants N needs, the high N removal rate of soybean and the lack of fertilizer amendment results in little to no net N benefit. Toomsan et al. (1995) found a small (15 kg ha^{-1}) N benefit resulting from a soybean crop, indicating that in certain circumstances there may be a small net benefit to the soil.

4.3.3 Carbon to Nitrogen Ratios

Carbon to nitrogen ratios for first year reference crops varied significantly between crop residues and site years (Table 4.15, Table 4.16, Table 4.17). There was a significant treatment by site year interaction so site years were analysed separately.

Table 4.15. Analysis of variance (ANOVA) of effect of crop (Crop) and site year (SiteYear) on C:N ratios of first year treatment crops.

ANOVA	P > F
Crop	<.0001
SiteYear	0.0014
Crop*SiteYear	<.0001

Table 4.16. Effect of preceding crop (Crop) and site year (SiteYear) on carbon to nitrogen (C:N) ratios of first year treatment crops.

Factor	Crop	C:N Ratio (Range)
Crop	Canola	54.1 **c** (40-71)
	Corn	85.6 **a** (69-112)
	Soybean	65.4 **b** (53-82)
	Wheat	41.3 **d** (22-56)

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Table 4.17. Carbon to nitrogen (C:N) ratios of preceding crop residues at harvest at Carman (2013,2014), Kelburn (2013, 2014) and Portage (2014).

	Carman		Kelburn		Portage
Treatment	2013	2014	2013	2014	2014
	----------C:N Ratio----------				
Canola	58.8 **b**	39.7 **c**	47.2 **c**	54.2 **c**	70.5 **b**
Corn	111.5 **a**	84.1 **a**	75.9 **a**	69.3 **b**	87.1 **a**
Soybean	52.6 **b**	57.3 **b**	60.0 **b**	82 **a**	75.2 **ab**
Wheat	33.1 **c**	53.5 **b**	22.1 **d**	41.9 **d**	55.9 **c**
P > F	<.0001	0.0002	<.0001	<.0001	0.0018

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Corn had the highest C:N ratio at four of five site years. Wheat had the lowest C:N ratio at three of five site years and canola had the lowest C:N ratio at two site years (Table 4.17). On average, corn produces more residue and has a higher C:N ratio than other crops such as canola and soybean (MAFRD, 2007). Research on C:N ratios in

Manitoba has shown that C:N ratios can vary significantly, especially for corn stover and wheat straw (MAFRD, 2007). Carbon to nitrogen ratios for corn stover can vary from 65-95:1. Carbon to nitrogen ratios for wheat straw can range from 35-85:1. Carbon to nitrogen ratios for soybean are approximately 65:1. Carbon to nitrogen ratios for canola are approximately 33:1 (Gan et al., 2011). Carbon to nitrogen ratios for canola varied greatly and were often higher than expected, ranging from 22-71:1. Carbon to nitrogen ratios for the other crops varied as well but were generally closer to expected averages (Table 4.16).

The high C:N ratio of corn residue can increase microbial activity to break residue down and consequently increase microbial uptake of soil N, decreasing soil mineral N (Brady and Weil, 2008). This decrease in soil mineral N could result in reduced spring soil test N, which can reduce yield as soil mineral N is important for early soybean growth before N fixation begins (Starling et al., 1998). There was, however, no significant effect of C:N ratio on yield (P=0.29)(Figure 7.4).

Carbon to nitrogen ratios of residues of preceding crops may have had an effect on spring soil test N, resulting in higher spring soil test N on canola stubble and lower spring soil test N on corn stubble. Crop residues with higher C:N ratios resulted in greater %Ndfa in the soybean crop the following year, although the relationship was not statistically significant when all site years were combined (P=0.11)(Figure 7.3). Carbon to nitrogen ratio did not have a significant effect on yield (P=0.29)(Figure 7.4) or mycorrhizal colonization (P=0.20)(Figure 7.5).

4.3.4 Effect of Arbuscular Mycorrhizal Fungi on Biological Nitrogen Fixation

We hypothesized in this experiment that the presence of AMF would positively impact BNF rates. There was a significant relationship between mycorrhizal colonization and BNF (Figure 4.8). Correlation analysis resulted in an R value of 0.52, indicating a moderate correlation between mycorrhizal colonization and BNF.

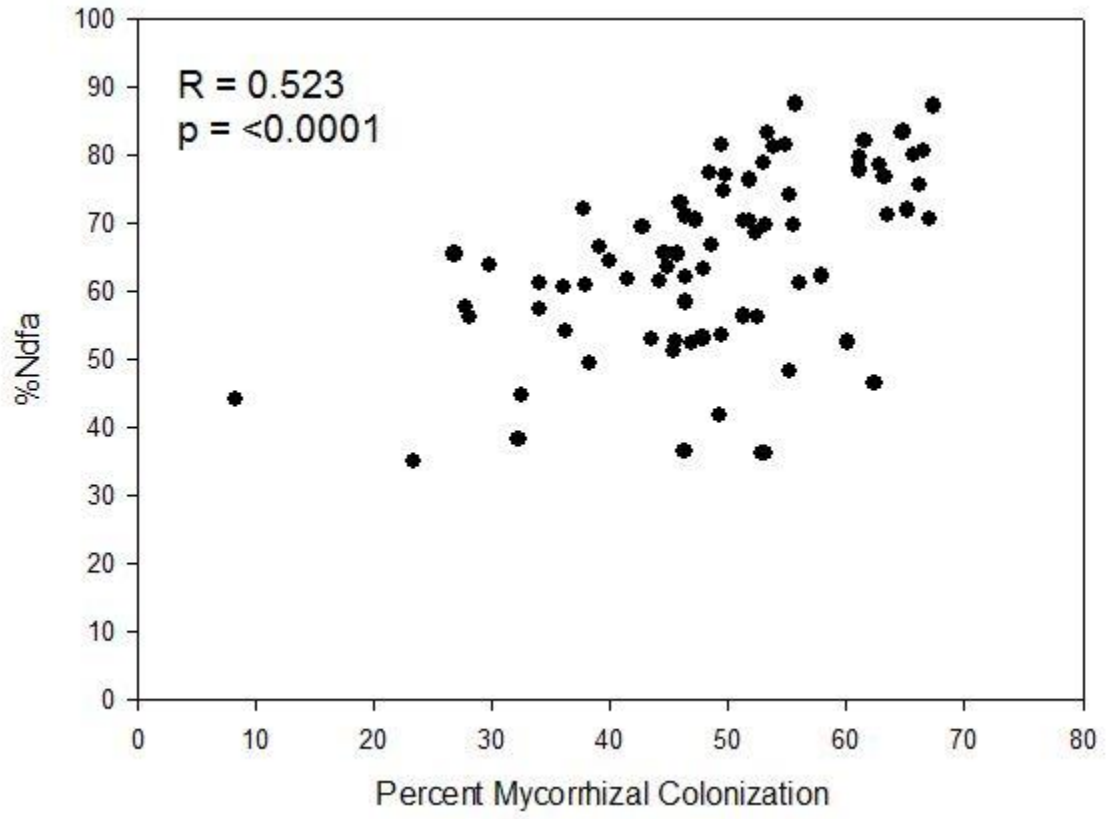

Figure 4.8. Correlation between mycorrhizal colonization and biological nitrogen fixation (%Ndfa) pooled across five site years.

Some studies have shown that the presence of AMF can positively impact BNF. Ruiz-Lozano et al. (2001) found that under drought conditions, soybean plants that had been inoculated with both *Glomus mosseae* and *Bradyrhizobium japonicum* had increased

acetylene reductase activity (a measure of BNF) by 112 percent, increased protein content by 15 percent, and increased leghemoglobin content by 25 percent, compared to plants that had only been inoculated with *Bradyrhizobium japonicum*. Plants inoculated with mycorrhizae and *Bradyrhizobium* also had increased nodule weight. Decreased oxidative damage to nodules likely produced these differences. The ability of AMF to increase water availability to plants under drought conditions likely improved nodule health, and under normal moisture conditions differences may not be as drastic. Nwoko and Sanginga (1999) found that inoculating soybean with certain combinations of AMF and *Bradyrhizobium* strains resulted in greater nodule weight than inoculating plants only with *Bradyrhizobium*. Other AMF strains did not increase nodule weight, indicating that different strains of AMF have varying degrees of impact on the *Bradyrhizobium* soybean symbiosis.

4.4 Soybean Plant Population

Residues of preceding crops did not have a significant effect on subsequent soybean plant populations (Table 4.18). Soybean plant populations varied between site years but not between crop treatments.

Table 4.18. Effect of preceding crop (Crop) and site year (SiteYear) on subsequent soybean plant populations (plants ha^{-1}).

Factor	Treatment	Plant Population (Plants ha^{-1})
Crop	Canola	490 000
	Corn	500 000
	Soybean	475 000
	Wheat	480 000
	Mean	485 000
SiteYear	Carman 2013	510 000 **b**
	Kelburn 2013	345 000 **d**
	Carman 2014	435 000 **c**
	Kelburn 2014	545 000 **b**
	Portage 2014	605 000 **a**
	Mean	485 000
ANOVA		P > F
Crop		0.4578
SiteYear		<.0001
Crop*SiteYear		0.3386

Means within a column followed by a different letter are statistically significant at P<0.05 according to Fisher's Protected LSD.

Plant stands ranged from 435 000 plants ha^{-1} at Carman in 2014 to 605 000 plants ha^{-1} at Portage in 2014. Several studies indicate that soybean yields well over a wide range of plant stands and adapts well to yield highly even in low plant stands (Carpenter and Board, 1997; Andrade and Abbate, 2005; De Bruin and Pedersen, 2008). This is a result of greater branch dry matter per plant, resulting in more branch nodes, branch reproductive nodes, and branch pods (Carpenter and Board, 1997). There was no significant effect of plant population on yield.

Residue management may have played a role in limiting the effect of treatment crop residues on the soybean test crop plant population and yield. All plots were cultivated once in the fall to incorporate treatment crop residues, and once in spring to

create a good seed bed for the soybean test crop. Due to the high amounts of residue, corn plots were also disced twice in the fall and once in the spring prior to establishment of the soybean test crop. Residue may have affected plant stands more if residue had not been managed. Corn residue has been shown to negatively affect yield in no-till cropping systems where residue is typically not managed with tillage (Wilhelm et al., 1986). A meta-analysis comparing no-till to conventional tillage in corn and soybean found that no-till management systems only result in lower soybean yield compared to conventional till systems on poorly drained soils, indicating that residue management may not have a significant effect on plant population or yield where excess moisture is not an issue (DeFelice et al., 2006). As a result we would not expect residue to have a significant effect on soybean plant stands in this study.

4.5 Conclusion

Preceding crop treatment had a significant effect on soybean yield, mycorrhizal colonization and biological nitrogen fixation. Preceding crop significantly affected soybean yield at two of five site years, with the soybean-soybean sequence resulting in significantly higher yield than all other treatments at one site year and significantly lower yield than all other treatments at another site year. Preceding crop treatment significantly affected mycorrhizal colonization, with the canola-soybean sequence consistently having lower levels of total mycorrhizal colonization than all other treatments. Preceding crop treatment also affected BNF, with the corn-soybean and soybean-soybean sequence often having higher levels of BNF than the wheat-soybean and canola-soybean crop sequences.

The results of this study have implications for producers in Manitoba. Soybean yield was fairly consistent among crop sequences, indicating that soybean may have a high degree of flexibility in Manitoba crop rotations. In some situations growing soybean following soybean may result in a loss of yield. Using survey data, Kubinec (2012) indicates that soybean following soybean yield 95 percent of the provincial average for soybean yield. Other studies have shown that growing soybean continuously over multiple growing seasons is more likely to result in a significant loss of yield.

The preceding crop was consistently found to affect soybean's symbiotic relationship with AMF and *Bradyrhizobium*. Growing soybean following canola or wheat resulted in reduced mycorrhizal colonization compared to soybean following soybean or corn. In this study, mycorrhizal colonization was shown to affect plant P concentration and to a small degree yield. This could potentially affect soybean yield in fields where P levels are critically low. Soybean following soybean and corn tends to fix more nitrogen biologically than soybean following wheat or canola. Although nitrogen fertilization is generally not recommended for soybean, this could affect soybean yield in fields where N levels are critically low.

The effect of preceding crop on C:N ratios and spring soil test N levels influenced BNF in subsequent soybean crops. Preceding crops with higher C:N ratios generally resulted in lower spring soil test N levels, likely as a result of N immobilization in the soil. The rate of fertilizer N applied to the preceding crop also affected spring soil test N levels. These factors encouraged increased BNF in the following soybean crop. Spring soil test P levels similarly influenced AMF colonization, with low levels of soil P encouraging higher levels of AMF colonization. Carbon to nitrogen ratios and P

fertilization of the previous crop, however, did not have a significant impact on spring soil test P and AMF colonization. These interactions can play a role in growers agronomic decisions, as the preceding crop may have a significant impact on spring soil test N, BNF and AMF colonization in the subsequent crop.

5.0 GENERAL DISCUSSION

5.1 Effect of Crop Sequence on Soybean Yield

The relatively new introduction of soybean to Manitoba means there is little literature available to understand how soybean can best be incorporated into existing Manitoba crop rotations. This experiment was designed to determine what effect a preceding crop would have on three variables of soybean production: yield, arbuscular mycorrhizal fungi (AMF) colonization and biological nitrogen fixation (BNF).

Crop sequence had an effect on yield at several sites, but not at others. The most striking results were in the soybean-soybean crop sequences, which resulted in greater yield than all other crop sequences at one site year and lower yield than all other crop sequences at another. The increased yield of the soybean-soybean sequence at Carman in 2013 was at least partially the result of volunteer soybean increasing yield. There were no differences in yield between any of the crop sequences at three other site years.

Manitoba Agricultural Services Corporation (MASC) compiles insurance data for large acre (>120 acres) fields in Manitoba. The most recent data available (2008-2012) shows that soybean grown on soybean yields 95 percent of the relative average soybean yield in Manitoba (Kubinec, 2012). Soybean following corn, wheat and canola yield above the relative average soybean yield in Manitoba (107, 103 and 101 percent, respectively). This could partially explain why the soybean-soybean sequence did not yield differently than other crop sequences at three of five site years. The high yield at one site year, and low yield at another site year, indicates that the soybean-soybean sequence can increase yield variability and may result in lower yields. Other factors, such as excellent growing conditions at Carman in 2013, and very wet and saturated soil

at Portage in 2014, likely contributed to the high and low yield of these two site years. The insurance data, however, does not indicate if soybean yield declines slightly in all fields where continuous cropping occurs, or if large declines in yield occur in a small number of fields while leaving others unaffected. The dynamics of soybean yield loss are, therefore, not well understood.

Soybean yield declines the longer it is in continuous rotation. Although some studies have shown a significant decline in soybean yield after two years of continuous soybean (Xu et al., 1999; Liu and Yu, 2000) other studies have shown little difference in yield after two consecutive years of soybean (Crookston et al., 1991; Porter et al., 1997). All of these studies, however, found increasing declines in soybean yield after three years, and a significant increase in yield from rotation. Unlike corn, where yield decline is most pronounced after two years of consecutive cropping and then stabilizes, soybean yield continues to decline the longer it is in monoculture (Crookston et al., 1991; Porter et al., 1997; Liu and Herbert, 2002; Davis et al., 2012). This experiment was only a two-year rotational study. Although one site year did have lower soybean yield following soybean, a study that examined three or more years of continuous soybean might find that yield declines even more the longer the rotation is in soybean.

5.2 Effect of Crop Sequence on Mycorrhizal Colonization

Crop sequence affected mycorrhizal colonization more noticeably and significantly than any other measurement. This effect was consistent across site years. The presence of a non-mycorrhizal crop (canola) significantly reduced mycorrhizal colonization at all site years after just one year. Soybean grown on crop stubble that was

mycotrophic (corn and soybean) had approximately 14 percent higher total AMF colonization than soybean grown on canola. Soybean grown on wheat also had significantly lower mycorrhizal colonization than soybean-corn or soybean-soybean. Previous research has confirmed the impact of mycorrhizal and non-mycorrhizal host plants on AMF colonization in a succeeding crop (Harinikumar and Bagyaraj, 1988; Douds et al., 1997; Gavito and Miller, 1998; Karasawa et al., 2002; McGonigle, 2009; Chen et al., 2013). This experiment confirms the tendency for increased mycorrhizal colonization when mycorrhizal crops, such as soybean, are grown on mycorrhizal crop stubble.

There was a small but significant relationship between AMF and soybean yield. Mycorrhizae have been shown to have the greatest impact on yield when soil P levels are very low (Lekberg and Koide, 2005). Fixen et al. (2010) reports that critical P levels for soils in the Northern Great Plains are about 20 ppm. All site years except Kelburn in 2014 had soil P levels below 20 ppm. This indicates that AMF could be an important supplier of P to the plant in these low P soils.

Soil P also significantly affected AMF colonization. There was a significant negative linear relationship between increasing soil P and decreasing AMF colonization. This confirms previous research (Miranda and Harris, 1994; Treseder, 2004; Carrenho et al., 2007) that shows that plants more readily form a symbiotic relationship with AMF when soil nutrients (especially P) are low. The large amount of plant energy required to support AMF means that if a plant can sufficiently supply itself with nutrients it will be less inclined to form the symbiotic relationship with AMF. On soils with high P levels or where P application has occurred for many years the impact of AMF may be considerably

less significant than on soils where P levels are low. AMF may play an important role in cropping systems where P levels are low or where P fertilization does not occur. In Manitoba, this may include organic systems or systems where P has been steadily depleted over a number of years. Soybean is sensitive to phosphorus and the recommended maximum application rate in Manitoba is just 10 lb/ac of P_2O_5 (MAFRD, 2007). In situations where soil P levels are low and P application levels are limited, AMF may play an important role in supplying the plant with necessary P for growth and strong yield.

5.3 Effect of Crop Sequence on Biological Nitrogen Fixation

Crop sequence significantly impacted the degree of BNF that occurred in the soybean plant. This was a result of the varying soil N levels and C:N ratios of each crop. Soil test N was often the highest in the canola-soybean sequence. Soil test N was often the lowest in the corn-soybean sequence. This resulted in lower levels of BNF as plants utilized soil available N instead of acquiring N through fixation due to the high energy cost of fixation (Peoples et al., 1995b). Part of the reason for lower N levels in the soybean-soybean sequence was due to the fact that no fertilizer N was applied to the soybean treatment crop in the first year, while treatment crops of corn, wheat and canola all received a nitrogen amendment in the first year of the crop sequence. Corn and soybean also have slightly higher N removal rates than canola, resulting in less residual N for the next crop (Table 4.11).

The C:N ratios of the preceding crops can affect nitrogen availability in the succeeding crop due to the potential for nitrogen immobilization. In this experiment a

significant effect of C:N ratio on soil N was only evident at two of the five site years. Soil microorganisms require nitrogen as they break down crop residues. In crops with a low C:N ratio, there is often enough nitrogen present in the residue to meet the microorganisms N requirements. In crops with higher C:N ratios, however, there is not enough N in the residue to supply the microorganisms. They immobilize residual soil N instead, rendering it temporarily unavailable to the following crop. The high C:N ratio of corn may have contributed to the corn-soybean sequence having the highest level of BNF at three of five site years. When soil microbes immobilize nitrogen, making it unavailable to the crop, the plant is more dependent on BNF to obtain nitrogen.

The soybean-soybean sequence resulted in the highest levels of BNF at two of five site years. The absence of nitrogen fertilizer amendments for both first year treatment crop soybeans and second year test crop soybeans likely resulted in this crop sequence being more dependent on biological nitrogen fixation. The second year soybean crop may also have benefited from the presence of soil rhizobia due to the previous year's inoculation, although most literature does not support the hypothesis that rhizobia from the previous year's inoculation contribute to BNF except in soils that have not been seeded to soybean in over five years (Triplett et al., 1993). Soil N, which is impacted by the C:N ratio of the previous crop, plays an important role in determining the degree of BNF in the subsequent soybean crop.

5.4 Effect of Mycorrhizal Colonization on Biological Nitrogen Fixation

Biological nitrogen fixation is an energy intensive process that consumes significant amounts of phosphorus through ATP consumption. It has been theorized that

improved plant P supply via AMF may increase the ability of the plant and rhizobia to perform BNF. Other studies have found that the presence of AMF may result in an increased release of organic root exudates from the host plant, which is often a nutrient source for rhizobacteria (Fitter and Garbaye, 1994). While some studies have found interactions between AMF and rhizobacteria to be symbiotic and mutually beneficial, others have found that these relationships can be competitive and deleterious. For example, some bacteria have been shown to improve fungal germination and growth rates, while others increase root cell permeability, improving the ability of plants to form a beneficial interaction with AMF (Artursson et al., 2006). Interactions are often unique and vastly different between species (Artursson et al., 2006).

The results of this experiment found a significant linear relationship between AMF colonization and BNF (R^2=0.27). Although AMF and BNF were found to be correlated, without understanding the underlying mechanisms of the relationship it is difficult to determine if this is a causal relationship or if other factors resulted in higher levels of both AMF and BNF, without a symbiotic relationship between AMF and *Bradyrhizobium*. Lower soil P levels are known to increase AMF colonization, while lower soil N results in increased BNF (Peoples et al., 1995b; Treseder, 2004). Nutrient depleted soils may simply encourage formation and colonization of AMF and *Bradyrhizobium*, without any specific interaction between the two species (Artursson et al., 2006). Increased AMF colonization in soybean roots, however, did not appear to result in a decrease in BNF by *Bradyrhizobium japonicum*. This indicates that there did not appear to be competition for nutrients between AMF and *Bradyrhizobium*.

5.5 Future Research

The recent expansion of soybean into Manitoba means there is much more research to be done concerning soybean in rotation. Of particular importance are long term crop rotation experiments that study the effect of various rotations over the course of multiple years. A two-year crop sequence, while useful, is limited in its ability to make long term predictions regarding the influence of crop sequence on yield, AMF colonization, and BNF. Long term rotational studies that look at the effect of soybean production on other crops, and vice-versa, will be important in order to understand the role and impact of soybean production in Manitoba.

Many other studies both in North America and around the world have documented the effect of declining yield with increasing years of monoculture soybean (Crookston et al., 1991; Porter et al., 1997; Xu et al., 1999). It is not known to what extent yield can be expected to decline after multiple years of continuous soybean in Manitoba. Trials comparing multiple years of continuous soybean to an annual two crop rotation as well as a diverse crop rotation will help to understand the effect of continuous rotations compared to annual and diverse rotations.

The relative lack of disease pressures in Manitoba compared to other regions with a long history of soybean production affords producers a significant advantage in terms of avoiding economic losses through disease and pests (MAFRD, 2014). This advantage is not likely to persist in the long term as new pests and diseases are introduced to Manitoba and disease pressure builds (Eathington et al., 1993). The most notable and damaging pest currently not found in Manitoba is soybean cyst nematode (SCN). Globally, SCN is responsible for more yield loss than any other disease or pest (Wrather

et al., 2001). Soybean cyst nematode also represents the greatest source of yield loss in the United States and Ontario. Soybean cyst nematode has been found in many counties in North Dakota and will likely be introduced to Manitoba at some point in the future. Crop rotation has been identified as the most effective agronomic management practice for controlling SCN (Ross, 1962; Noel and Edwards, 1996; Porter et al., 2001). This and other disease and pest challenges will require new research to understand how these diseases and pests will impact soybean production in Manitoba and how their impact can be mitigated as much as possible.

5.6 Conclusion

The introduction of soybean into Manitoba presents growers with new economic and agronomic opportunities. The ability to expand beyond the wheat-canola rotation that has been so prevalent on the Canadian Prairies offers producers a unique chance to expand and diversify their crop rotations. This has the potential to improve soybean yield as well as the yield of other crops in rotation as well.

Crop rotation is one of the oldest agronomic management practices known. It is an integral component of good agronomic management and plays an important role in improving soil fertility, soil structure, reducing incidence of disease, weeds and pests, and increasing yield (Bullock, 1992). Despite the widespread use of commercial fertilizers and pesticides, crop rotation remains an important tool for producers to maximize agronomic output and maintain the sustainability of the agroecosystem. In this experiment we have found that crop rotation can have a significant impact on soybean

yield, AMF colonization and BNF by *Bradyrhizobium*. In soils with low nutrient status, AMF and BNF are critical in order for soybean plants to obtain adequate nutrients.

The role of AMF and BNF in crop production and their potential to improve yield and soil quality has been documented for many years. The ability of AMF and *Bradyrhizobium* to provide the plant with otherwise unavailable sources of nutrients is an important component of soybean production. These soil microorganisms will play an increasingly important role as producers shift to farming systems that are increasingly dynamic, profitable and sustainable.

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7.0 Appendix

Table 7.1. Mean soybean yield by site year averaged over all treatments compared to rural municipality (RM) mean yield for that year and RM ten-year long term average (LTA) from 2005-2015.

Site Year	Mean Yield	RM Average Yield†	% of RM Average	RM LTA†
	----------Kg ha^{-1}----------			
Carman 2013	3748	2683	140	2676
Carman 2014	3093	2529	122	2676
Kelburn 2013	2679	2764	97	2562
Kelburn 2014	2945	2367	124	2562
Portage 2014	2390	2307	104	2643

†Source: Manitoba Agricultural Services Corporation (2015).

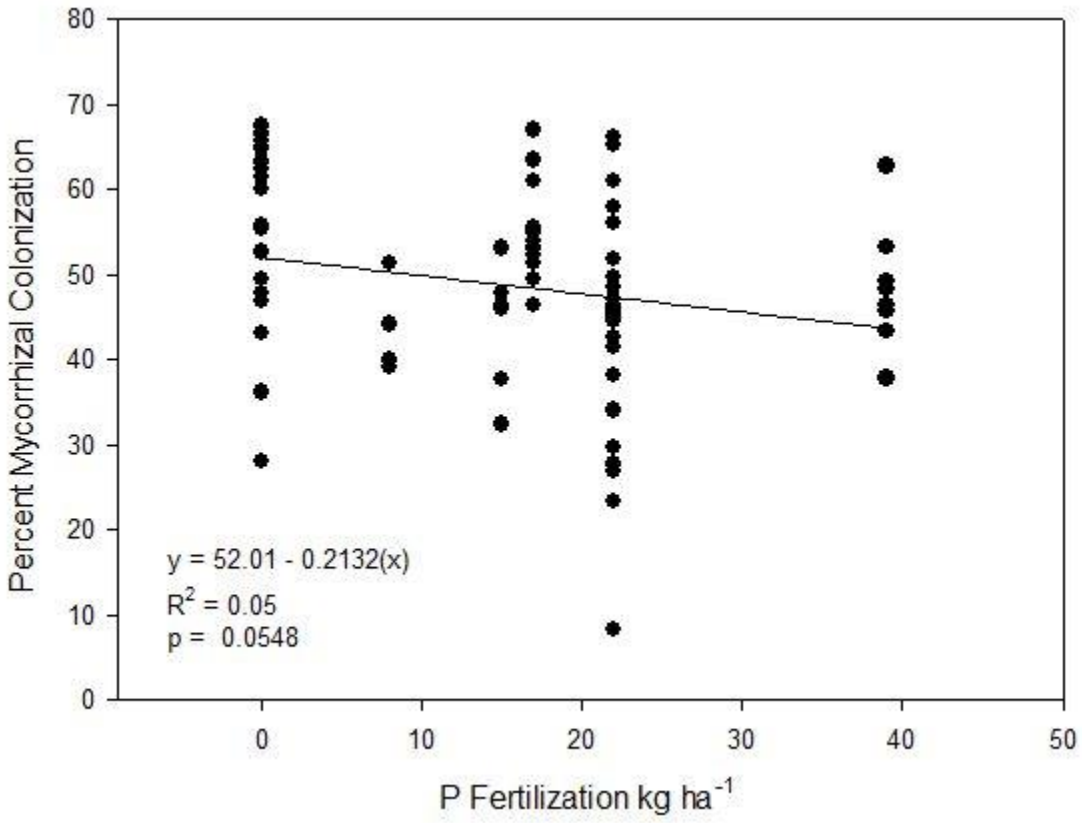

Figure 7.1. Effect of P fertilizer rates of preceding treatment crop on mycorrhizal colonization of soybean test crop roots at the V3 stage.

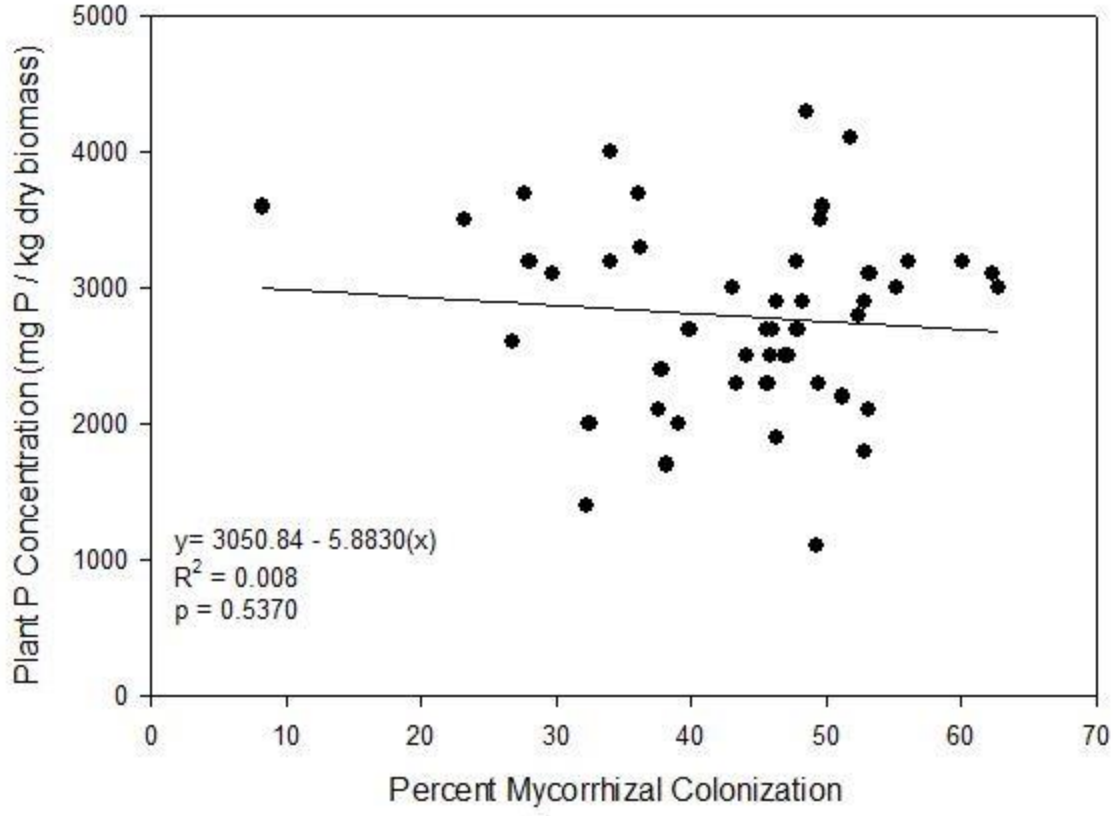

Figure 7.2. Effect of AMF colonization on soybean shoot plant P concentration at V3 stage.

Table 7.2. Effect of crop (Crop) and site year (SiteYear) on crop biomass at harvest for year 1 treatment crop at Carman, Kelburn and Portage in 2013.

Factor	Treatment	Biomass (Kg ha^{-1})
Crop	Canola	4040 **b**
	Corn	11690 **a**
	Soybean	2700 **c**
	Wheat	3990 **b**
SiteYear	Carman	5440
	Kelburn	4810
	Portage	6570
ANOVA		P > F
Crop		<.0001
SiteYear		0.0002
Crop*SiteYear		0.0014

Table 7.3. Crop biomass at harvest for year 1 treatment crops that were soil incorporated at Carman, Kelburn and Portage in 2013 prior to growing the soybean test crop.

Treatment	Carman	Kelburn	Portage
		----------Kg ha^{-1}----------	
Canola	2670 **c**	4050 **b**	5410 **b**
Corn	11390 **a**	9950 **a**	13740 **a**
Soybean	3200 **c**	2660 **c**	2240 **c**
Wheat	4490 **b**	2590 **c**	4900 **b**
Mean	5440	4810	6570
P value			
Crop	<.0001	<.0001	<.0001

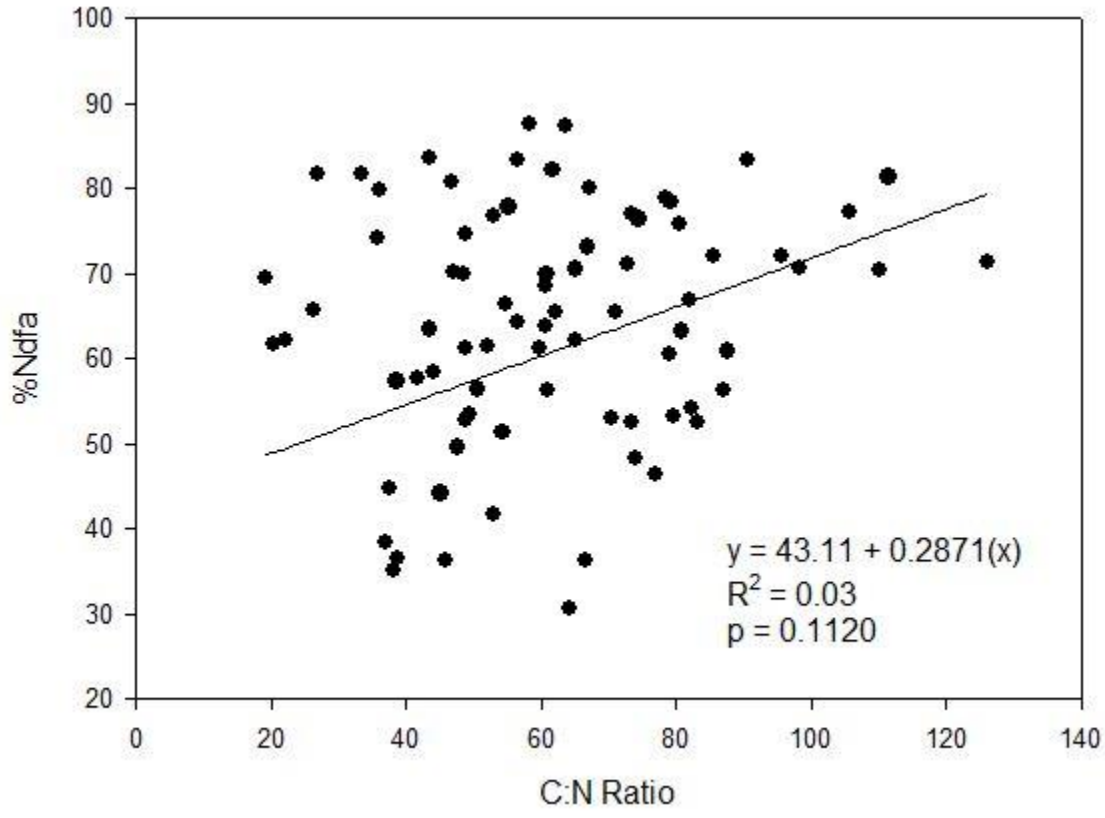

Figure 7.3. Effect of carbon to nitrogen ratio of preceding crop on %Ndfa in subsequent soybean crop.

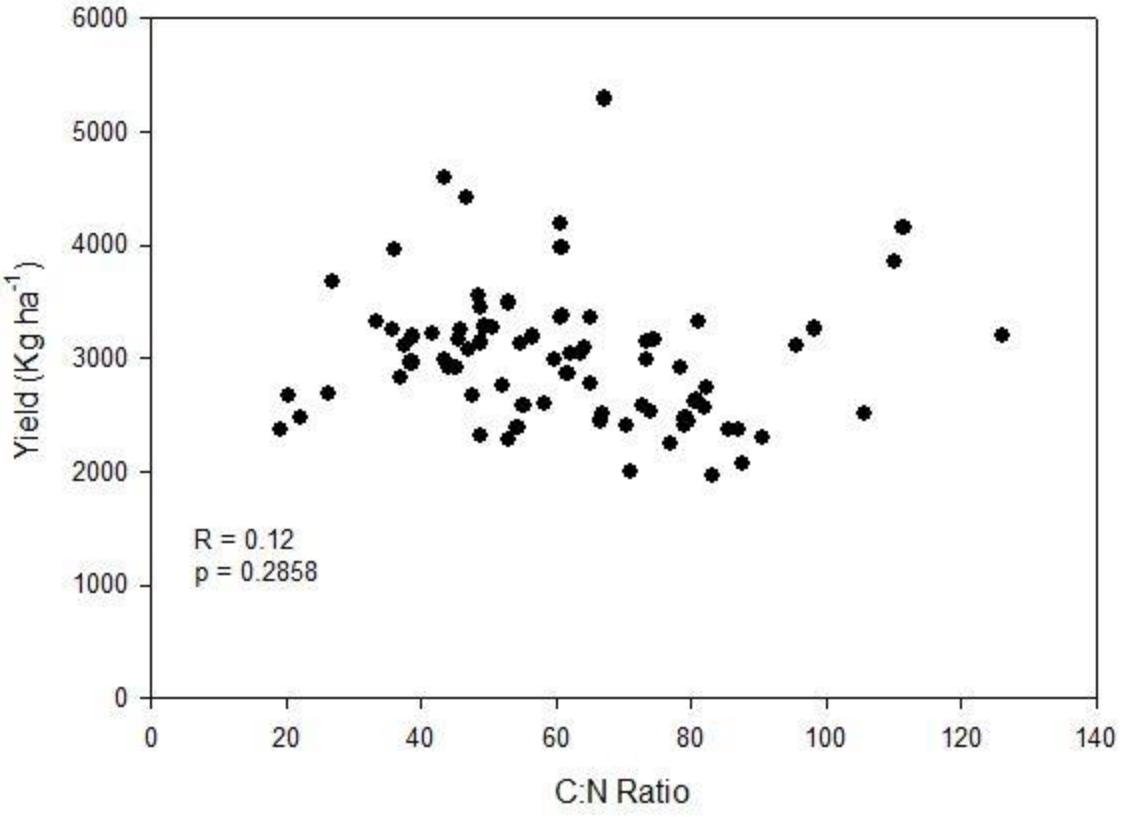

Figure 7.4. Correlation between C:N ratio of preceding crop and soybean yield.

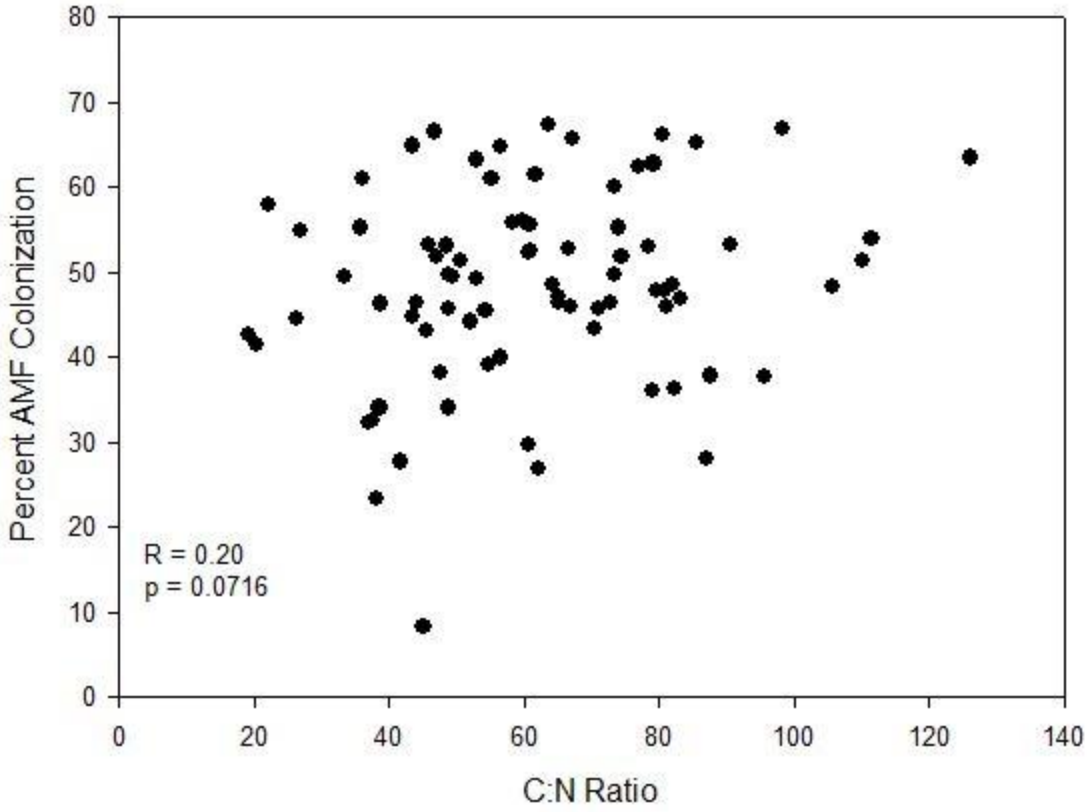

Figure 7.5. Correlation between C:N ratio of preceding crop and colonization of soybean roots by AMF.