NITROGEN FIXATION BY ANNUAL LEGUME GREEN MANURES IN A SEMI-ARID CROPPING SYSTEM

by

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ABSTRACT

There is renewed interest within agriculture to improve and sustain soil fertility. Legume crops can provide an alternative nitrogen (N) input to a cropping system through biological N fixation. The contribution of N from legume cropping systems depends on the quantity of N fixed and soil processes that influence soil N availability. The primary objectives of this project were 1) to evaluate the effect of planting and termination time on biomass production and N fixation by two legume green manure crops; and 2) to investigate the role of soil P availability on legume growth and N fixation.

A two-yr dryland field study was conducted with three treatments: legume (field pea and lentil), planting time (spring and summer [2010 only]), and termination time (flower, intermediate [2009 only], and pod). Two methods, ^{15}N natural abundance and N difference, were used to quantify N fixation. In 2009, N fixed by spring-planted lentil was higher by pod than flower (P=0.03). There was no difference in N fixed by spring-planted pea among termination times, likely because of reduced precipitation during the middle of the growing season. In 2010, both spring-planted crops fixed more N by pod than flower (P<0.01) and more N was fixed by spring-planted crops than summer-planted crops (P<0.01).

A greenhouse study was conducted in an unsterilized, low P soil (8 mg kg-soil^{-1}) with three treatments: legume crop (field pea or lentil), P fertilizer (0, 4, or 8 mg P kg^{-1}), and arbuscular mycorrhizae fungus (AMF) inoculum (AMF-, AMF+). Shoot biomass was sampled at flower, and N fixation was estimated with ^{15}N natural abundance method. Fertilization increased biomass yield and tissue N and P uptake for both crops (P<0.01) and increased N fixed by pea (P<0.01). Inoculation with AMF had little effect on measured parameters; however, there was an increase in pea biomass and N uptake in the AMF+ versus AMF- treatments at the 4P rate.

Several variables that affect N fixation in semi-arid cropping systems were identified in this project, however further research assessing the effect of other soil and environmental conditions on N fixation and the cycling of fixed N in an agroecosystem is needed.

CHAPTER 1

INTRODUCTION

Background

A central tenet of sustainable agriculture is to improve and sustain soil fertility and productivity with fewer anthropogenic inputs (Peoples et al. 1995). This is particularly true for the management of nitrogen (N), the nutrient that most limits plant growth in agricultural cropping systems. Commercial N fertilizer is the most heavily relied upon input in conventional cropping systems in the United States (U.S.) and globally (Gruber and Galloway 2008), and its use has increased steadily over the last 50 years (Service 2008). This increase in synthetic fertilizer use, along with other agricultural advancements, has led to immense productivity and profitability within the agricultural industry. Yet, these gains have come at a cost to air and water quality (Tilman et al. 2001), and it is projected that further use along the same growth trajectory will lead to substantial negative changes in the global environment (Vance 2001). Furthermore, recent volatility in the costs of both energy and N fertilizer has caused concern about the future of synthetic fertilizers as a cost-effective means of producing nutrient-intensive crops (Huang 2009). Consequently, interest in more energy-efficient cropping systems and alternative N inputs is increasing within the scientific, agricultural, and health communities both globally and throughout the U.S.

An alternative N input that is gaining renewed attention in conventional agriculture is biological N fixation. Biological N fixation refers to the bacteria-mediated

process of fixing atmospheric dinitrogen (N_2) gas to a reduced form of N that can be used by plants and microorganisms. For most temperate cropping regions, the most applicable way of utilizing biologically fixed N is with the incorporation of legumes. Through a symbiotic relationship with root-associated N-fixing bacteria, collectively termed rhizobia, legumes are able to incorporate fixed N into their biomass. Prior to the onset of synthetic fertilizer use in the mid-twentieth century, N fixation via the legume-rhizobia symbiosis was a key component of successful long-term agriculture; evidence of N fixation in cropping systems extends back thousands of years to ancient civilizations in Asia, Africa, and the Americas (McNeill and Winiwarter 2004). While N fixation remains an integral component of some modern agriculture systems, such as organic and soybean systems, there is a growing interest regarding its potential as an alternative N source in conventional agriculture, including cropping systems in the northern Great Plains (NGP).

Legume-Based Cropping Systems in the NGP

Historically, the majority of cropland in the semi-arid NGP, including Montana, has been managed with conventional tillage and cereal crop-fallow systems. The adoption of reduced tillage practices in recent years, and the associated improvements in soil water storage, has given rise to new opportunities for diversifying traditional cropping systems, including legume crop rotations (Padbury et al. 2002; Zentner et al. 2004). For example, legume acreage in Montana increased 3.4-fold between 2004 and 2010, with most production occurring in the northeast part of the state (Montana Agriculture Statistics

Service 2010). Adoption of legume-based crop rotations can provide a multitude of agronomic and ecological benefits, including a break in disease and pest cycles, improvement in soil quality, and an additional N source to the system (Kirkegaard et al. 2008). In organic systems, legumes are integral for maintaining soil fertility and are often the primary N input to the system (Miller et al. 2011). For conventional systems, legumes in rotation can partially offset N fertilizer inputs by 1) eliminating the need to add N fertilizer during the legume rotation, and 2) cycling a portion of fixed N remaining in legume residues into the system where it can become available for subsequent non-legume crops (Zentner et al. 2001). A reduction in N fertilizer inputs can have considerable economic, energetic, and environmental gains for an agroecosystem (Pimentel et al. 2005). Because N fertilizer represents one of the most costly and energy-intensive inputs to a cropping system (Piringer and Steinberg 2006), reducing fertilizer N inputs can substantially decrease the energy inputs of a cropping system (Burgess et al. in review; Zentner et al. 2011). Environmental pollution problems, such as nitrate (NO_3) leaching into water systems and increased nitrous oxide (N_2O) emissions into the atmosphere, have also been attributed to excess N fertilizer use (Motavalli et al. 2008; Powers 2007).

Legume-based crop systems can also play an important role in the short- and long-term cycling of soil N and carbon (C) in an agroecosystem. Legume residues contain a large quantity of N and typically have a lower C:N than cereal residues (Janzen and Kucey 1988). Initially, this can result in a faster release of mineralized residue N to the system that is available for the subsequent crop. Yet, depending on soil and

environmental conditions, a large proportion of legume residue N can be immobilized into a more recalcitrant form of organic N which can enhance the long-term N-supply of the soil (Drinkwater et al. 1998). Because of the inherent complexity of soil nutrient dynamics, the contribution of legumes to soil N and C cycling in an agroecosystem can vary widely and is often difficult to assess (Walley et al. 2007). Further evaluation of long-term crop rotation studies is needed to more fully understand the effect of legume-based systems on soil N and C over time, particularly in comparison to monoculture wheat or rotations without legumes.

Legume Green Manures

Not all areas in the NGP and Montana are able to include grain legumes in rotations without substantially impacting subsequent yields, primarily due to limited soil water. For these areas, short-duration legume green manure (LGM) crops have been suggested as a beneficial option for diversifying crop systems dependent on fallow (Brandt 1996; Miller et al. 2006; Zentner et al. 2001). Legume green manures are crops grown with the intent of returning all biomass and subsequently fixed N and C to the soil. Unlike legumes grown for grain, LGM crops do not need to be grown to full maturity and can be managed to conserve soil water. Thus, LGM crops as a partial summer-fallow replacement may fill a unique niche in many NGP and Montana dryland cropping systems.

Research in organic systems in Montana has shown positive effects on subsequent crop yields following an LGM crop (Izard 2007; Miller et al. 2011). Yet, unlike organic producers that are entirely reliant on LGMs and other non-synthetic sources to provide

their nutrient needs, conventional producers are interested in LGMs as an additional tool in their N management, not necessarily as a replacement. This is an important distinction because crop management tactics and efficient use of resources are often quite different between the two systems and little research has been done evaluating LGMs in conventional agriculture (Zentner et al. 2004). To fully assess the N benefits associated with LGMs in the NGP, and specifically Montana, an estimate of the amount of N fixed by the legume crop as well as the effects of agronomic and management practices on N fixation is needed. To my knowledge, N fixation by a LGM crop has not directly quantified in Montana.

Soil Fertility Effects on Legume Production in the NGP

Legume crops have an increased requirement for certain nutrients, partly due to the role of specific nutrients in the N fixation symbiosis (Chalk 2000; Chalk et al. 2010). This is particularly the case for phosphorus (P), the second most limiting nutrient after N. Nitrogen fixation is an energy and P-intensive process requiring approximately 16 mol of ATP for 1 mol of N_2 fixed (Ruiz-Lozano et al. 2001). Phosphorus is also required for proper formation and function of nodules (O'Hara 2001), the organelles where N fixation takes place. Thus, soil P deficiencies not only limit legume growth and yields, but also the amount of N fixed by the crop (Cadisch et al. 1993; Israel 1987).

Many soils in the NGP have low soil P availability as a result of high soil calcium levels. For example, approximately 70% of Montana soils and 86% of North Dakota soils test low to moderate for soil available P (Fixen et al. 2011). Despite this, NGP legume producers may not be adding P during the legume rotation (Burgess et al. in review) and

research investigating the effect of soil P deficiencies on legume crop production in the NGP is limited. Identifying agronomic practices that improve P availability in NGP soils could have a positive effect on legume-based cropping systems in the region.

Quantifying N Fixation

Both of the main studies of this project required direct measurement of N fixation by a legume crop. A number of methods have been developed to measure symbiotic N fixation; the choice of a particular method depends on the type and site of the experiment, the available resources, and species and/or system in question (Gathumbi et al. 2002). The acetylene-reduction assay method is useful for quantifying N fixation and establishing peak N fixation rates at a given time; however, it does not integrate N fixation over a growing period, a common requirement for many N fixation studies, including those in this project. For time-integrated estimates of N fixation, an N balance or ^{15}N-isotopic method is recommended (Unkovich et al. 2008).

The simplest form of the N balance method is to grow an N-fixing crop and an adjacent non-N-fixing (reference) crop and assume the difference in plant tissue N at harvest between the two crops is the quantity of N fixed. Although this method is inexpensive and easy to carry out in a field setting, it has been shown to be highly inaccurate and unreliable in that it largely under- or over-estimates the effect of soil N in the system (Rennie 1984). A recommended alternative to the N balance method is the N difference (ND) method in which available soil N levels under both the legume and reference crops are taken into account. By incorporating the soil N component, soil N

transformations over the growing season and respective differences in N uptake from the two crops can be assessed, assuming soil N transformations and losses are equal between the crops. Another assumption of the ND method is that root N between the two crops is similar. Since it is not practical to effectively harvest roots from field plants, shoot N:root N ratios are assumed to be similar between the crops. This assumption is difficult to validate in field settings and root N, including losses of root N to the soil, may represent a large pool of fixed N that is ignored in N fixation estimates (Sawatsky and Soper 1991; Walley et al. 2007).

Methods for quantifying N fixation with ^{15}N include isotope dilution (ID) and natural abundance (NA). These methods can be advantageous over the N balance methods as they provide a yield-independent estimate of N fixation (Chalk et al. 2000). Both ^{15}N-isotopic methods rely on the naturally-occurring ^{15}N abundance in the atmosphere to quantify N fixation. Atmospheric ^{15}N concentrations are a uniform 0.3663 atom% globally (Shearer and Kohl 1986), but because of N transformation processes that preferentially discriminate for or against ^{15}N, soil and biological material tend to have ^{15}N concentrations different than the atmosphere. The change in ^{15}N concentrations with respect to atmospheric ^{15}N is expressed as $\delta^{15}N$ in parts per thousand (‰); hence, the $\delta^{15}N$ of atmospheric N is 0‰. Many soils become enriched in ^{15}N over time due to microbial discrimination against the heavier ^{15}N isotope in favor of the lighter ^{14}N isotope. The degree of soil ^{15}N enrichment in a field can vary considerably and is influenced by a number of physical and biochemical factors (Hauggaard-Nielsen et al. 2010). The ID method can partially adjust for this variability by adding a known quantity

of plant available ^{15}N to the system, usually with ^{15}N-labeled fertilizer, and including a non-N-fixing reference crop. The ID method was most widely used in the 1970s-1990s, prior to advancements in isotopic mass spectrometry in the 1980s, that led to development of the NA method (Unkovich et al. 2008). The ID method may still be favorable for soils where the $\delta^{15}N$ of plant-available soil N is less than 2‰ or high-precision mass spectrometry analysis is not available. The NA method quantifies N fixation by calculating the difference between a non-N fixing reference plant and a N-fixing legume that is obtaining N from both the soil and atmosphere, after accounting for isotopic fractionation between ^{14}N and ^{15}N in the aboveground shoot of the legume (the *B* value). The first assumption of the NA method is that the legume and reference plant are accessing the same pools of soil N. This assumption requires that the two plants are grown near one another and also that the two plants have similar stature and rooting morphology. A second assumption is that there is either no discrimination or identical discrimination between ^{14}N and ^{15}N in the plants' uptake and metabolism of N, or any discrimination has been accounted for.

The ND and NA methods were selected to measure N fixation in this project based on their relative simplicity, each method's use of different N cycling processes, and expected accuracy in soils with low available N, where dependence on N fixation was expected to be high (Unkovich et al. 2008). Also, because the two methods have similar methodologies, they could be carried out simultaneously in the field experiment, creating an opportunity to increase the robustness of N fixation quantification and compare the two methods with respect to assumptions and spatial variation. Spatial and temporal

variability in N fixation is expected and it is recognized that both N fixation and the methods that aim to quantity it are sensitive to numerous environmental and soil factors that may cause a high degree of inaccuracy and/or imprecision in N fixation estimates (Unkovich et al. 1994; Walley et al. 2001). The emphasis for the two main studies of this project was not on actual quantities of N fixed, but rather the effect of experimental treatments on N fixation.

Project Objectives

The goal of the first two studies of this thesis project was to evaluate agronomic and management factors affecting biomass production and N fixation by legume crops in the NGP. The first study, a 2-yr field experiment, assessed the effect of planting time and termination time on biomass production and N fixation by pea and lentil green manure crops in a conventional cropping system (Chap. 2). The second study, a greenhouse experiment, evaluated the role of soil P availability on legume growth and N fixation as affected by P fertilization and AMF inoculation (Chap. 3). The goal of the third study was to evaluate soil N and C data from two long-term crop rotation studies to assess the role of legume-based cropping systems on soil N and C content dynamics over time (Appendix A).

References

Brandt SA (1996) Alternatives to summerfallow and subsequent wheat and barley yield on a dark brown soil. Can J Plant Sci 76 (2):223-228.

Burgess M, Miller PR, Jones CA (in review) Pulse crops improve energy intensity and productivity of cereal production in Montana, USA. J Sustainable Agric.

Cadisch G, Sylvester-Bradley R, Boller BC, Nosberger J (1993) Effects of phosphorus and potassium on N-2 Fixation (N-15-Dilution) of field-grown *Centrosema acutifolium* and *C. macrocarpum* Field Crops Res 31 (3-4):329-340.

Chalk PM (2000) Integrated effects of mineral nutrition on legume performance. Soil Biol Biochem 32 (4):577-579.

Chalk PM, Alves BJR, Boddey RM, Urquiaga S (2010) Integrated effects of abiotic stresses on inoculant performance, legume growth and symbiotic dependence estimated by N-15 dilution. Plant Soil 328 (1-2):1-16.

Economic Research Service (2008) U.S. consumption of nitrogen, phosphate, and potash, 1960- 2007. http://www.ers.usda.gov/Data/FertilizerUse/

Fixen PE, Bruulsema TW, Jensen TL, Mikkelsen R, Murrell TS, Phillips SB, Rund Q, Stewart WM (2011) The fertility of North American soils, 2010. Better Crops, vol 94.

Gathumbi SM, Cadisch G, Giller KE (2002) N-15 natural abundance as a tool for assessing N-2-fixation of herbaceous, shrub and tree legumes in improved fallows. Soil Biol Biochem 34 (8):1059-1071.

Gruber N, Galloway JN (2008) An Earth-system perspective of the global nitrogen cycle. Nature 451 (7176):293-296.

Hauggaard-Nielsen H, Holdensen L, Wulfsohn D, Jensen ES (2010) Spatial variation of N-2-fixation in field pea (*Pisum sativum* L.) at the field scale determined by the N-15 natural abundance method. Plant Soil 327 (1-2):167-184.

Huang W (2009) Factors contributing to the recent increase in U.S. fertilizer prices, 2002-08. Agricultural Resources Situation and Outlook vol AR-33, U.S. Department of Agriculture ERS. http://www.ers.usda.gov/Publications/AR33/AR33.pdf.

Israel DW (1987) Investigation of the role of phosphorus in symbiotic dinitrogen fixation. Plant Physiol 84 (3):835-840.

Izard EJ (2007) Seeking sustainability for organic cropping systems in the northern Great Plains: Legume green manure management strategies. Montana State University, Bozeman, MT

Janzen HH, Kucey RMN (1988) C, N, and S mineralization of crop residues as influenced by crop species and nutrient regime. Plant Soil 106:35-41.

Kirkegaard J, Christen O, Krupinsky J, Layzell D (2008) Break crop benefits in temperate wheat production. Field Crops Res 107 (3):185-195.

McNeill JR, Winiwarter V (2004) Breaking the sod: Humankind, history, and soil Science 304:1627-1629.

Miller PR, Engel RE, Holmes JA (2006) Cropping sequence effect of pea and pea management on spring wheat in the northern Great Plains. Agron J 98 (6):1610-1619.

Miller PR, Lighthiser EJ, Jones CA, Holmes JA, Rick TL, Wraith JM (2011) Pea green manure management affects organic winter wheat yield and quality in semiarid Montana. Can J Plant Sci 91 (3):497-508.

Motavalli PP, Goyne KW, Udawatta RP (2008) Environmental impacts of enhanced-efficiency nitrogen fertilizers. Crop Management. doi:10.1094/CM-2008-0730-02-RV

O'Hara GW (2001) Nutritional constraints on root nodule bacteria affecting symbiotic nitrogen fixation: a review. Aust J Exp Agr 41 (3):417-433.

Padbury G, Waltman S, Caprio J, Coen G, McGinn S, Mortensen D, Nielsen G, Sinclair R (2002) Agroecosystems and land resources of the northern Great Plains. Agron J 94 (2):251-261.

Peoples MB, Herridge DF, Ladha JK (1995) Biological Nitrogen-Fixation - An Efficient Source of Nitrogen for Sustainable Agricultural Production? Plant Soil 174 (1-2):3-28.

Pimentel D, Hepperly P, Hanson J, Douds D, Seidel R (2005) Environmental, energetic, and economic comparisons of organic and conventional farming systems. Bioscience 55 (7):573-582.

Piringer G, Steinberg LJ (2006) Reevaluation of energy use in wheat production in the United States. J Ind Ecol 10 (1-2):149-167.

Powers SE (2007) Nutrient loads to surface water from row crop production. Int J Life Cycle Assess 12 (6):399-407.

Rennie RJ (1984) Comparison of N balance and N-15 isotope-dilution to quantify N-2 fixation in field-grown legumes. Agron J 76 (5):785-790.

Ruiz-Lozano JM, Collados C, Barea JM, Azcon R (2001) Arbuscular mycorrhizal symbiosis can alleviate drought-induced nodule senescence in soybean plants. New Phytol 151 (2):493-502.

Sawatsky N, Soper RJ (1991) A quantitative measurement of the nitrogen loss from the root-system of field peas (*Pisum Avense* L) grown in the soil. Soil Biol Biochem 23 (3):255-259.

Shearer G, Kohl DH (1986) N-2 fixation in field settings - estimations based on natural N-15 abundance. Aust J Plant Physiol 13 (6):699-756.

Tilman D, Fargione J, Wolff B, D'Antonio C, Dobson A, Howarth R, Schindler D, Schlesinger WH, Simberloff D, Swackhamer D (2001) Forecasting agriculturally driven global environmental change. Science 292 (5515):281-284.

Unkovich M, Herridge DF, Peoples MB, Cadisch G, Boddey RM, Giller KE, Alves B, Chalk PM (2008) Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR Monograph. Australian Centre for International Agricultural Research, Canberra, AU.

Unkovich MJ, Pate JS, Sanford P, Armstrong EL (1994) Potential precision of the delta-N-15 natural-abundance method in-field estimates of nitrogen-fixation by crop and pasture legumes in south-west Australia. Aust J Agric Res 45 (1):119-132.

Vance CP (2001) Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. Plant Physiol 127 (2):390-397.

Walley FL, Clayton GW, Miller PR, Carr PM, Lafond GP (2007) Nitrogen economy of pulse crop production in the northern Great Plains. Agron J 99 (6):1710-1718.

Walley FL, Fu GM, van Groenigen JW, van Kessel C (2001) Short-range spatial variability of nitrogen fixation by field-grown chickpea. Soil Sci Soc Am J 65 (6):1717-1722.

Zentner RP, Basnyat P, Brandt SA, Thomas AG, Ulrich D, Campbell CA, Nagy CN, Frick B, Lemke R, Malhi SS, Fernandez MR (2011) Effects of input management and crop diversity on non-renewable energy use efficiency of cropping systems in the Canadian Prairie. Eur J Agron 34 (2):113-123.

Zentner RP, Campbell CA, Biederbeck VO, Miller PR, Selles F, Fernandez MR (2001) In search of a sustainable cropping system for the semiarid Canadian prairies. J Sustainable Agric 18 (2-3):117-136.

Zentner RP, Campbell CA, Biederbeck VO, Selles F, Lemke R, Jefferson PG, Gan Y (2004) Long-term assessment of management of an annual legume green manure crop for fallow replacement in the Brown soil zone. Can J Plant Sci 84 (1):11-22.

CHAPTER 2

NITROGEN FIXATION BY PEA AND LENTIL IN A SEMI-ARID AGROECOREGION: EFFECT OF PLANTING AND TERMINATION TIMING

Introduction

For semi-arid dryland cropping systems of the northern Great Plains (NGP), water and nitrogen (N) are often the most limiting factors for crop production. Crop-fallow systems continue to dominate in a few areas of the region, including much of Montana, to increase soil water for alternate year crop production. Although fallow has reduced some of the agronomic risk introduced by highly variable precipitation patterns that characterize the NGP, it has been identified as one of the least sustainable cropping practices in the region because of its effects on soil erosion and degradation (Brandt 1996; Larney et al. 1994; Zentner et al. 2001). Additionally, recent volatility in fertilizer prices has increased interest in reducing off-farm inputs and specifically N inputs. In response to these concerns, a short-season legume green manure (LGM) crop grown as a partial-fallow replacement crop has been suggested as a viable alternative to full fallow periods, primarily due to the unique ability of legumes to biologically fix N from the atmosphere (Pikul et al. 1997; Zentner et al. 1996).

The addition of biologically fixed N and biomass through LGM crops can have a number of ecological and agronomic benefits for an agroecosystem. These may include an increase in soil organic matter, disruption of pest cycles, and an increase in the availability of soil N for subsequent crops (Kirkegaard et al. 2008). In turn, LGMs can provide a partial offset of fertilizer N and a reduction in the energy intensity of the system

through lower fertilizer inputs (Pimentel et al. 2005; Zentner et al. 2011). To fully assess the potential of these benefits to an agroecosystem, an estimation of the amount of N fixed by the LGM crop is required. A number of studies have estimated N fixation by legume crops grown to full maturity in the NGP (Bremer et al. 1988; Gan et al. 2010; Rennie 1984; Rennie and Dubetz 1986), yet to our knowledge, direct quantification of N fixation by LGM crops in Montana has not been documented.

Decisions regarding the management of LGM crops in semi-arid agroecosystems are influenced by strategies that best use and conserve soil water. These decisions include legume species, planting time, and termination time. In general, annual legumes such as pea (*Pisum sativum*) and lentil (*Lens culinaris*) provide a better tradeoff between water use and N contribution than perennial legumes (Biederbeck et al. 1996). Plant growth habit (fall or spring planting) can affect the amount of stored soil water used by the crop by altering the timing of crop development. Miller et al. (2011) showed a soil water advantage for winter pea over spring pea due to winter pea maturing earlier in the growing season, allowing for an earlier termination date and less drawdown of soil water. However, fall-seeded crops may be more susceptible to poor germination and weather-induced crop loss than spring varieties (Chen et al. 2006). A novel practice proposed for reducing inputs in reduced tillage LGM systems is to ‘summer-seed’ an LGM crop and allow the crop to terminate naturally (e.g., frost or drought) in the fall. Very little research has been done investigating summer-seeded LGMs in dryland cropping systems, and its effects on N fixation and water use are relatively unknown.

Termination timing decisions of LGMs require a compromise between maximizing N fixation and using stored soil water. Peak N fixation by legume crops has been observed to occur in the period from flower to early pod development (Jensen 1987). However, this growth period often corresponds with peak crop water use and subsequent drawdown of stored soil water (Bremer et al. 1988). A 12-yr research study by Zentner et al. (2004; 2006) at Swift Current, SK found that, for the first 6 yr, May-seeded lentil terminated at full flower had detrimental effects on subsequent wheat crops due to soil water depletion. For the following 6 yr, lentil was seeded in April and terminated at the first bud stage. This management change increased subsequent wheat yields, resulting in higher net revenue for LGM-wheat over fallow-wheat in a conventional cropping system. Research in Montana also found that terminating at early flower stage was beneficial over pod stage due to increased water use by the latter stage causing reduced yields in the following crop (Izard 2007; Miller et al. 2006). Nonetheless, there are situations (e.g., years of high in-crop precipitation) when extending the growth period could be advantageous to maximize N fixation amounts with less effect on soil water.

Two well-established methods for estimating symbiotic N fixation over the growing period are the N difference (ND) and ^{15}N natural abundance (NA) methods. The ND method compares the difference in plant N uptake and available soil N between an N-fixing plant and a neighboring non-N-fixing (reference) plant, with the difference assumed to be fixed N. The NA method relies on differences in the naturally-occurring abundance of isotopic-^{15} N between the atmosphere and the soil-plant system. The

amount of N fixed is calculated by the differences in ^{15}N abundance between the N-fixing legume obtaining N from both the soil and atmosphere and a non-N-fixing reference plant accessing N wholly from the soil. Isotopic fractionation of ^{15}N in the legume shoots is adjusted for by measuring the ^{15}N abundance of aboveground legume tissue grown under N-free conditions (*B* value). Proper selection of a reference plant is a key component for the NA method and variability because of reference plant characteristics can cause substantial differences in estimates of N fixation (Nyemba and Dakora 2005; Pate et al. 1994). Including more than one reference plant, such as in-field weeds, has been suggested to increase the accuracy of the method (Boddey et al. 2000; Unkovich et al. 2008). The underlying assumptions, advantages, and disadvantages associated with these methods have been extensively reviewed (Hardarson and Danso 1993; Peoples et al. 2002; Unkovich and Pate 2000).

The primary objective of this study was to assess the effect of planting time and termination time on biomass production and N fixation by spring pea (*P. sativum* cv. Arvika) and spring lentil (*L.* culinaris cv. Richlea). Because N fixation estimates can vary among methods, our objective was to focus more on determining whether there were significant effects of planting and termination time on N fixation rather than absolute quantities of N fixed. A secondary objective was to compare N fixation estimates by the ND and NA methods and assess their suitability for estimating N fixation in a semi-arid cropping system.

Materials and Methods

Field Description and Experimental Design

This 2-yr plot study was conducted in 2009 and 2010 at two different sites (1.4 km apart) on the same commercial farm near Amsterdam, MT (45^{o} 45' N, 111^{o}23' W). Each site was established on barley (2009) or wheat (2010) stubble with at least 3 yr of previous no-till management. Soil types were Amsterdam silt loam (frigid Typic Halpustoll) and Brocko silt loam (frigid Aridic Calciustept) for 2009 and 2010, respectively. Soil properties for both sites are summarized in Table 2.1. Growing season precipitation was measured at each site with a tipping bucket rain gauge (Hobo, Onset Corp., Bourne, MA). Over-winter precipitation, monthly mean high temperature averages, and long-term average (LTA) data were gathered from the nearest meteorological station located 19 km away in Belgrade, Montana.

The study was a randomized complete block design consisting of four blocks. Plots were 8 by 12 m. Main plot factors were 1) legume species (pea or lentil); 2) planting time; and 3) termination time. Planting times were winter (W), spring (Sp), or summer (Su); however, W plots failed to fully establish in both years and were not included in this study. Termination timings were flower (50% of plants had one open flower), intermediate (7 d after flower; 2009 Sp only), pod (50% of plants had one flat-pod), or natural crop senescence due to drought or frost, whichever came first (2009 Su only). Drought-induced senescence of naturally-terminated Su crops in 2009 resulted in a large loss of plant tissue N and data were not reported. In 2010, Su crops were terminated at flower and pod. In both years, a 2-m strip of spring wheat (*Triticum aestivum* cv.

Choteau) was planted adjacent to each legume plot to serve as a non-N-fixing reference crop.

Crop Management, Field, and Laboratory Measurements

Agronomic management factors for both years are given in Table 2.2. Fungicide-treated seed was sown directly into stubble with a seed row width of 0.25 m (2009) and 0.3 m (2010). Commercial granular rhizobia inoculum (Optimize Pulse IF, EMD Crop Bioscience, Brookfield, WI) was applied with the legume seeds (2.8 kg ha^{-1}). Nitrogen fertilizer was mid-row banded and PKS fertilizer was placed with the seed. All crops, with the exception of natural, were terminated with glyphosate sprayed at a rate of 0.63 kg ha^{-1} a.e. plus 3 kg ha^{-1} of ammonium monosulfate. Weed and insect management practices for both years were reported by Burgess et al. (in prep).

Soil was sampled from four locations within each legume plot (near the center of each quadrant) at both seeding and harvest and at two locations within the wheat strip at harvest. Soil cores were extracted to a depth of 0.9 m, split into 0.3-m depth increments, and composited by depth. Samples were stored in plastic-coated paper bags and placed in coolers for transport from the field to laboratory. Aboveground legume and wheat biomass was harvested 3 d after glyphosate application to account for continued crop water use following application. Biomass was collected by clipping crop plants at the soil surface from two rows of approximately 0.5-m width and 1-m in length, repeated at the front and back of each legume plot and wheat strip. Biomass was composited in the field for a total collection area of approximately 1-m^2 from each plot and wheat strip. If present, at least two plants of the two dominant non-N-fixing weeds in each plot were

collected to increase the number of reference plants. In 2009, *Bromus tectorum*, a weedy grass with both winter and spring growth habits, was collected from 20 of 24 Sp plots; weeds were absent from all pod-terminated Sp pea plots. In 2010, both *B. tectorum* and *Sisymbrium altissimum*, a broadleaf mustard, were collected from all Sp plots (n=16), and either *Kochia scoparia* or *Amaranthus retroflexus* (both broadleaf weeds) were collected from 12 of 16 Su plots.

In the laboratory, soils were weighed, dried (50^{o}C, 72 h), and reweighed to determine bulk density and gravimetric water content. Dried soil was ground (<2 mm) and subsamples were extracted for soil NO_3-N with 1 M KCl as outlined by Bundy and Meisinger (1994). Extracts were analyzed using a flow injection analyzer (Lachat Instruments Inc., Loveland, CO). Soil bulk density values were averaged by depth across all plots from the same sampling date and used to calculate volumetric water content from gravimetric water content values and soil NO_3-N content (kg N ha^{-1}) from soil NO_3-N concentrations. Aboveground biomass was dried (50^{o}C, 72 h), weighed, and ground (<0.5 mm) and tissue subsamples were analyzed for total N and isotope-^{15}N determination with a continuous flow isotope ratio mass spectrometer (Stable Isotope Facility, UC Davis).

Nitrogen Fixation

Nitrogen fixation was estimated with ND and NA methods. Wheat was used as the reference crop for both methods and collected in-plot weeds were used as an additional reference crop for the NA method. Nitrogen fixed by the ND method was calculated as reported by Unkovich et al. (2008):

$$\text{N fixed} = \left(\text{Plant N}_{\text{legume}} - \text{Plant N}_{\text{wheat}}\right) + \left(\text{Soil N}_{\text{legume}} - \text{Soil N}_{\text{wheat}}\right) \quad [1]$$

It was assumed soil NO_3-N at seeding was the same for both the legume plot and reference wheat strip and soil N in Eq. 1 refers to soil NO_3-N at harvest. Nitrogen fixation by the NA method was calculated separately using both wheat (NA-wheat) and weeds (NA-weed) as reference plants. The proportion of N derived from the atmosphere via fixation (%Ndfa) was calculated as reported by Shearer and Kohl (1986):

$$\%\text{Ndfa} = \left(\frac{\delta^{15}\text{N}_{\text{reference}} - \delta^{15}\text{N}_{\text{legume}}}{\delta^{15}\text{N}_{\text{reference}} - \text{B}}\right) * 100 \quad [2]$$

where δ^{15}N is:

$$\delta^{15}\text{N } (‰) = \left(\frac{\text{atom\%N}_{\text{sample}} - \text{atom\%N}_{\text{atmosphere}}}{\text{atom\%N}_{\text{sample}}}\right) * 1000 \quad [3]$$

B in Eq. 2 refers to the δ^{15}N of lentil (-0.71) and pea (-0.73) grown under N-free conditions and were determined by a sand-culture greenhouse experiment (Appendix B). These values are within the range of previously published B values reported for field pea and lentil cultivars (Unkovich et al. 2008). Total aboveground N fixed was calculated by multiplying biomass yield, tissue N concentration, and %Ndfa/100.

Statistical Analyses

All statistical analyses were performed using JMP 8.0 statistical software (SAS Institute, 2010). Analysis of variance (ANOVA) was performed to determine significant treatment differences and interactions at $P \leq 0.05$. Treatment differences were further evaluated using planned orthogonal contrasts and Fisher's protected least significant difference (LSD) tests. Blocks (replicates) were considered a random effect and, when applicable, planting time, termination time, legume species, and year were fixed effects.

Site years were analyzed independently for overall treatment effects on pea and lentil because of differences in planting and termination treatments between years. To determine the effect of year and legume species on measured variables, site years were combined for Sp crops (2009 intermediate-termination data excluded) and analyzed using ANOVA. Correlation analyses were performed to compare quantities of N fixed among N fixation methods. Paired t-tests were used to assess differences in reference plant $\delta^{15}N$ values collected from the same plot.

Results and Discussion

Climatic Conditions

Mean precipitation and temperature for the two years of this study and the 30-yr LTA are shown in Table 2.3. In 2009, precipitation during the spring growing season (May to mid-July) was below normal with May rainfall accounting for less than 50% of the monthly LTA. In 2010, spring growing season precipitation was above normal, particularly in June in which precipitation was 32% higher than the LTA. Summer precipitation in 2010 was marked by below average rainfall in July and near normal rainfall in Aug., excluding a large rain event that occurred post-harvest of Su pod treatments in late August. Mean monthly temperatures from May-Aug. 2009 were consistently 1-2^{o}C higher on average than the LTA. Mean monthly temperatures throughout the growing season in 2010 were more similar to the LTA.

Legume Biomass and N uptake

Biomass yield and shoot N uptake differed among termination times in both 2009 and 2010 and between planting times in 2010 (Table 2.4). In 2009, pea and lentil biomass increased by 65% and 140%, respectively, from flower to pod stage. Similar increases were observed for Sp crops in 2010 when pea and lentil biomass increased 55% and 159%, respectively, from flower to pod stage. For Su plots (2010), only lentil biomass increased between flower and pod. Averaged across termination timings, biomass production by Su pea and lentil crops were 50% and 38% less than yields for Sp pea and lentil, respectively, as a result of limited precipitation during the Su growing season. Shoot N uptake by Sp lentil was greatest at pod stage in both years and corresponded with increased biomass production. For Sp pea, the effect of termination time on shoot N differed between years. Shoot N uptake leveled off between flower and pod in 2009, yet increased between flower and pod in 2010.

A strong positive correlation between biomass production and N uptake has been reported for both lentil and pea (Biederbeck et al. 1996; Unkovich et al. 2010); however, the rate of N accumulation by legumes, either by N fixation or soil N uptake, has been shown to decline during early reproductive stages and can be related to water stress (Jensen 1987; Salon et al. 2001). Less consistent precipitation patterns in 2009, particularly in late June and early July, may have contributed to the cease in pea N uptake after flower. Izard (2007) also reported that spring pea shoot N did not differ between flower and pod and attributed it to scarce rainfall between the two stages.

Comparison of N Fixation Methods

Nitrogen fixation for pea and lentil as estimated by the ND method and NA-wheat and NA-weed methods resulted in a range of N fixed values for each treatment combination (Table 2.5). In both years, the ND method generally resulted in the highest N fixation estimates, whereas the NA-wheat method consistently resulted in the lowest estimates of N fixed. Estimates of N fixed by the NA-weed method were lower than the ND method in 2009 and similar to the ND method in 2010. Averaged across the two years and legume species mean estimates of N fixed for Sp crops were within 15 kg N ha-1 between the ND and NA-weed methods and differed by more than 39 kg N ha-1 between the ND and NA-wheat methods.

Although differences in the amount of N fixed were observed among methods, treatment effects on N fixation were often in agreement among the three methods and always in agreement for the ND and NA-weed methods. A regression analysis of N fixed by the two NA methods and the ND method revealed a significant positive linear relationship between methods in 2009 and 2010 (Fig 2.1). In both years, the NA-weed method showed a stronger association with the ND method than the NA-wheat method. In addition, there was better agreement among methods in 2010 than 2009, indicating site conditions were a factor affecting N fixation estimates among methods.

Estimates of N fixation by the ND method were believed to be more reliable in this study than estimates by the NA method, particularly the NA-wheat method. A main assumption of the ND method is that soil N processes are similar between the legume plot and adjacent reference plant plot. Crop water use exceeded precipitation in both years of this study (Appendix D) and soil N losses (e.g., volatilization, denitrification,

and leaching) were presumed to be relatively minimal under the semi-arid conditions in this system. Soil N mineralization was not directly measured in this study; however, relatively small differences in soil water content between the legume and wheat strip at each harvest (Appendix D) and low levels of soil organic carbon (<1.5%) suggest N mineralization rates between the legume plot and wheat strip were likely not large. Also, the ND method has been shown to work well in soils with low available N in which dependency on N fixation by the legume is expected to be high (Danso 1995). Averaged across the two years, available soil NO_3-N at seeding only represented 32% and 26% of N uptake by Sp lentil and pea, respectively, at pod, strongly suggesting that a high degree of N fixation by the legume crops was occurring.

Often cited problems associated with the NA method in field applications include the spatial and temporal variability of the ^{15}N abundance of soil available N (Hauggaard-Nielsen et al. 2010; Walley et al. 2001), and small observed differences between atmospheric and soil ^{15}N uptake (Bremer et al. 1993). Both of these issues apparently affected the accuracy and precision of the NA-wheat method in this study. For example, in 2009, the NA-wheat method resulted in 5 of 24 plots with negative amounts of N fixed (legume $\delta^{15}N$ > wheat $\delta^{15}N$). By comparison, the NA-weed method resulted in only one negative N fixed value in 2009 and N fixed values were always positive for the ND method. The high proportion of negative N fixation estimates by the NA-wheat method in 2009 was likely the result of high spatial variability in $\delta^{15}N$ of available soil N at the site as well as poor separation in $\delta^{15}N$ values between the legume and wheat crops (Appendix Table D.3.). The cause of spatial heterogeneity in the $\delta^{15}N$ signature of soil N pools and

its effect on N fixation by the NA method is difficult to identify (Androsoff et al. 1995; Hauggaard-Nielsen et al. 2010). In 2010, N fixation values were positive for all methods and there was less within-treatment variance in N fixation values by both NA methods, suggesting ^{15}N abundance of soil available N was more uniform at this site.

Precision of the NA method can be affected by the choice of reference plant (Dakora et al. 2008). A $\delta^{15}N$ enrichment of at least 2‰ difference between the reference plant and N-fixing plant has been recommended to meet acceptable precision of N fixation estimates by the NA method (Unkovich et al. 1994). In both years of this study, mean differences in $\delta^{15}N$ values between wheat and legumes at each harvest were less than 2‰ and wheat $\delta^{15}N$ values were consistently less than weed $\delta^{15}N$ values (Table 2.6). Subsequently, the NA-weed method resulted in higher observed N fixed values and greater method precision than the NA-wheat method, particularly in 2010 when mean $\delta^{15}N$ differences between broadleaf weeds and both legumes consistently exceeded 2‰. A study adjacent to the 2009 site used a non-nodulating pea isoline as the reference crop for the NA method and average $\delta^{15}N$ values were similar to *B. tectorum* (weed) $\delta^{15}N$ values at the 2009 site (Appendix C). Differences in $\delta^{15}N$ values among reference species have been observed in a number of studies utilizing the NA method (Bremer et al. 1993; Hauggaard-Nielsen et al. 2010; Houngnandan et al. 2008). For example, Pate et al. (1994) found nine different reference plants grown in the same field to differ in $\delta^{15}N$ content by more than 2‰ in some cases. Factors causing these differences have been attributed to different isotopic discrimination among species, dissimilar rates of plant N uptake throughout the growing season, and different rooting patterns. These are all possible

reasons for the observed differences in N fixation by the NA-wheat and NA-weed methods in this study and further emphasize the importance of reference plant selection when utilizing the NA method.

Treatment effects on N fixation

Regardless of potential inaccuracies by the ND and NA methods to quantify N fixation, the primary objective of this work was to evaluate treatment effects on N fixation. Treatment effects on N fixed as determined by the ND and NA-weed methods were consistently in agreement for both years of this study, strongly suggesting that the measured differences were real. For ease of interpretation and reasons stated above, N fixation amounts are only discussed for the ND method.

In 2009, termination timing had a significant effect on N fixed by lentil, but not pea. Nitrogen fixed by lentil more than doubled between flower and pod. In contrast, N fixed by pea was not different among the three terminations in 2009. In 2010, there was a significant termination effect on N fixation for both Sp crops. Lentil and pea fixed 65 and 30 kg ha^{-1} more N by pod than flower, respectively. For Su crops, there were no differences in N fixed between flower and pod. Planting time affected the amount of N fixed in 2010 with Sp crops fixing considerably more N than Su crops in three of the four treatments. The exception was lentil terminated at flower stage which fixed similar amounts of N in Sp and Su, resulting in a significant planting time by termination time interaction. Mean soil NO_3-N levels in the top 0.9 m at Su were less than Sp soil NO_3-N levels and nodulation by Su crops was likely not delayed by high levels of available soil N. Rather, decreased biomass production and subsequently poor N fixation by the Su

crops were likely a result of low precipitation and higher evapotranspiration during the Su growing season.

There is a well-established correlation between biomass production and N fixation for a number of leguminous crops (Unkovich et al. 2010). This relationship was observed for lentil in both years of this study and one year for pea. In 2009, Sp pea terminated at intermediate and pod stages appeared to have experienced enough water-stress to cease N fixation, yet not biomass production. Nitrogen fixation has been reported to be more sensitive to water-stress than biomass production and N assimilation for a number of legume crops, and can be affected by both the severity and timing of precipitation (Castellanos et al. 1996; Kirda et al. 1989; Serraj et al. 1999). A two-year, multisite dryland study in Saskatchewan found N fixation by pea in a drought year to be reduced by 40% on average across two sites compared to N fixed at the same sites in the following year with more normal precipitation (Bremer et al. 1988). Kirda et al. (1989) found a mild (single-cycle) water stress period decreased N fixation in soybean by almost 60% on average compared to non-water stressed plants, though biomass production was not different between the treatments. The timing of water stress relative to crop development can also influence N fixation (Thomas et al. 2004). This may have partially caused N fixation by pea to be more affected by water stress than lentil N fixation in Sp 2009.

When 2009 and 2010 Sp data sets were combined, year had a significant effect on N fixation ($P<0.001$). For pea, cooler and wetter growing conditions in spring 2010 led to greater biomass production and crop N demand, which in turn increased the amount of N

fixed by pod by approximately 80% compared to 2009. Lentil biomass and the amount of N fixed were similar between years at both flower and pod. In addition, there was a significant legume species effect on N fixation when 2009 and 2010 Sp data were combined ($P<0.001$). With the exception of pod-terminated pea in 2009, pea fixed more N than lentil at each termination time. The greatest difference in N fixed between species occurred at flower stage with pea fixing 1.4 and 3.4-fold more N than lentil in 2009 and 2010, respectively. Thus, in this system, pea was a better crop for fixing N than lentil, especially when terminated at flower stage.

Conclusions

Choosing when to plant and terminate a LGM crop can affect biomass production and the amount of N fixed by the crop and is influenced by in-crop growing season conditions. In this 2-yr field study, spring-planting resulted in substantially higher biomass yield and quantity of N fixed for pea and lentil compared to summer-planting as a result of reduced precipitation during the Su growing season. Winter LGM crops failed to establish in this study and further research will be needed to assess the effect of fall-planting on N fixation. Pod termination resulted in peak biomass production and N fixation for both legume crops when spring precipitation was above normal. Yet, when growing season precipitation was below normal and less consistent, N fixed by pea did not increase beyond flower stage despite an increase in biomass. Findings from this research indicate that planting a LGM prior to a period of high in-crop precipitation and terminating at pod stage can increase N fixation, but only when growing conditions are

optimal for the specific legume crop. In growing seasons with below normal or inconsistent precipitation patterns, early termination may result in similar N fixation amounts as a later termination. Not only would there be no additional N fixation gain from a later termination, but also a greater effect on soil water content, particularly in a drier year.

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Table 2.1. General soil properties for 2009 and 2010 study sites measured at spring seeding. Soil characteristics are for 0-0.15 m depth, unless otherwise stated.

Soil property	2009	2010
Soil texture	Clay loam/loam	Silt loam
Soil pH	6.6-8.0	7.9-8.4
Organic carbon (g kg^{-1})[a]	15	14
Olsen P (mg kg^{-1}) [a]	30	18
K (mg kg^{-1}) [ab]	486	561
SO_4-S (mg kg^{-1}) [a]	8	11
Spring NO_3-N (kg N ha^{-1})[ac]	29	46
Summer NO_3-N (kg N ha^{-1})[ac]	na[d]	33

[a] Analysis averaged across 12 composited samples of four subsamples per site.
[b] Ammonium acetate K.
[c] Measured to 0.9-m depth.
[d] Not reported for 2009 summer planting.

Table 2.2. Agronomic factors for 2009 and 2010 sites.

Agronomic Factor	2009	2010
Spring soil sample date	19-21 Apr.	2, 7 Apr.
Spring soil water[a], mm	221	183
Spring seeding date	13 Apr.	24 Mar.
Spring-seeded harvest date		
Flower	1 July	30 June
Intermediate	10 July	--
Pod	17 July	14 July
Summer soil sample date	17 June	21 June
Summer soil water[a], mm	223	246
Summer seeding date	22 June	21 June
Summer-seeded harvest date		
Flower	12 Aug.	11 Aug.
Pod (Natural in 2009)	14 Sep.	27 Aug.
Seeding rate, seeds m^{-2}		
Pea	80	80
Lentil	120	120
Wheat	250	250
Fertilizer[b] (kg ha^{-1})		
Pea, lentil	6-29-28-10	6-29-28-10
Wheat	11-58-56-19	6-29-28-10

[a] Plant available soil water (mm) to 0.9 m measured in stubble at or near seeding.

[b] N-P-K-S was applied as a granular 50/50 blend of monoammonium phosphate (11-52-0) and potassium sulfate (0-0-50-17); 2009 spring seeded wheat reference crop strips received double fertilizer due to seeder overlap.

Table 2.3. Precipitation and temperature data for Amsterdam, Montana, 2009 and 2010 sites.

	Precipitation (mm)			Mean temperature (^{o}C)		
	2009	2010	LTA[a]	2009	2010	LTA[a]
Oct.-Apr.[b]	115	110	154	0.8	-1.0	-1.1
May	26	58	57	12.7	9.6	10.5
June	63	86	65	15.4	15.7	15.1
July	56 (25)[d]	18 (13)[d]	29	19.9	19.6	18.9
Aug.	0	57 (22)[e]	29	19.5	18.9	18.3
Sep.	0	30	35	17.2	14.9	12.5
Growing season[c]	145	249	220	16.9	15.7	15.1

[a] Long-term average, 1971-2000 mean annual precipitation from the Western Regional Climate Center, Desert Research Institute, Reno, NV.
[b] Over-winter preceding the growing season.
[c] May-Sept.
[d] Monthly precipitation total through spring pod harvest (17 July 2009 and 14 July 2010).
[e] Monthly precipitation total through summer pod harvest (27 Aug. 2010).

Table 2.4. Means and summary of analysis of variance for aboveground dry biomass and shoot N uptake by legume species and year for each planting and termination treatment. Columns with the same letter are not statistically different and significant effects are shown in bold (P<0.05).

Planting[a]	Termination	Biomass (Mg ha^{-1})		Shoot N (kg ha^{-1})	
		Pea	Lentil	Pea	Lentil
		----------2009----------			
Spring[a]	Flower	3.0c	1.6b	105a	51b
	Int[b]	3.7b	2.5b	98a	72b
	Pod	4.9a	3.7a	107a	106a
$LSD_{0.05}$		*0.7*	*1.1*	*NS*[c]	*30*
Source of variation	*df*		*P* values		
Termination (T)	2	**<0.01**	**<0.01**	0.66	**0.01**
		----------2010----------			
Spring	Flower	3.2b	1.2bc	116b	45b
	Pod	5.0a	3.2a	147a	98a
Summer	Flower	1.8c	1.1c	47c	30b
	Pod	2.2c	1.8b	44c	39b
$LSD_{0.05}$		*0.7*	*0.6*	*22*	*21*
Source of variation	*df*		*P* values		
Planting (P)	1	**<0.01**	**<0.01**	**<0.01**	**<0.01**
T	1	**<0.01**	**<0.01**	0.08	**<0.01**
P x T	1	**0.01**	**<0.01**	**0.03**	**<0.01**

[a] Only spring-planted treatments were analyzed in 2009.
[b] Intermediate termination time.
[c] Not significant.

Table 2.5. Means and summary of analysis of variance for nitrogen (N) fixed as determined by the N difference (ND) and ^{15}N natural abundance (NA) methods by year and legume species for each planting and termination treatment. Columns with the same letter are not statistically different and significant effects are shown in bold (P<0.05).

Planting[a]	Termination	N fixed (kg N ha^{-1})					
		ND		NA-wheat		NA-weed[c]	
		Pea	Lentil	Pea	Lentil	Pea	Lentil
		----------2009----------					
Spring[a]	Flower	84.0a	35.2b	47.4a	21.7b	53.9a	16.4b
	Int[b]	74.3a	49.6ab	18.8a	13.4b	62.5a	29.2b
	Pod	75.2a	74.3a	21.0a	46.2a	na[d]	61.3a
	$LSD_{0.05}$	*NS*[e]	*24.8*	*NS*	*14.7*	*NS*	*32.0*
Source of variation	*df*	*P values*					
Termination (T)	2	0.63	**0.02**	0.13	**<0.01**	0.57	**0.03**
		----------2010----------					
Spring	Flower	106.0b	24.6b	78.6a	20.8ab	95.0b	31.0b
	Pod	135.7a	89.1a	85.6a	35.0a	123.8a	72.9a
Summer	Flower	32.2c	12.9b	26.5b	17.1b	43.5c	17.9b
	Pod	26.5c	20.3b	30.2b	30.0ab	35.4c	32.0b
	$LSD_{0.05}$	*28.7*	*16.7*	*17.0*	*16.5*	*21.8*	*23.5*
Source of variation	*df*	*P values*					
Planting (P)	1	**<0.01**	**<0.01**	**<0.01**	0.41	**<0.01**	**<0.01**
T	1	0.21	**<0.01**	0.34	**0.03**	0.20	**<0.01**
P x T	1	0.08	**<0.01**	0.76	0.90	**0.04**	0.06

[a] Only spring-planted treatments were analyzed in 2009.
[b] Intermediate termination time.
[c] Predominant weed reference crop for each year and planting were as follows: 2009/spring, *Bromus tectorum*; 2010/spring: *Sisymbrium altimissum;* 2010/summer: *Kochia scoparia* and *Amaranthus retroflexus*.
[d] Not available due to absence of weeds in these plots.
[e] Not significant.

Table 2.6. Mean tissue $\delta^{15}N$ (‰) for legumes, wheat, and collected weeds in 2009 and 2010.

Planting	Termination	Pea	Lentil	Wheat	Weed[a]
		----------2009----------			
Spring	Flower	0.49	0.51	1.51	1.58
	Int	0.57	0.78	0.98	2.27
	Pod	0.80	0.32	1.13	1.89
		----------2010----------			
Spring	Flower	-0.13	0.19	1.34	2.90
	Pod	-0.09	0.36	1.11	3.60
Summer	Flower	-0.34	-0.04	0.55	3.31
	Pod	-0.36	-0.31	0.71	2.18

[a] Weed species were *Bromus tectorum* (2009), *Sisymbrium altissimum* (2010 spring), and *Kochia scoparium* or *Amaranthus retroflexus* (2010 summer).

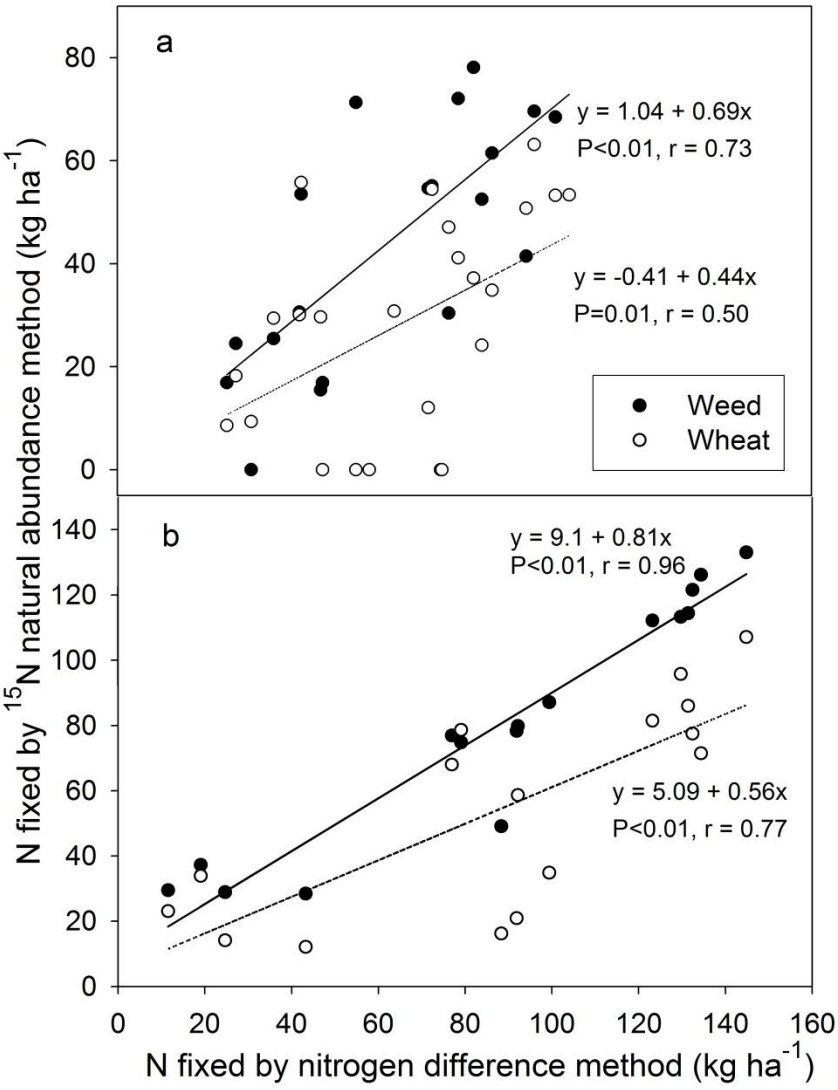

Figure 2.1. Correlation between nitrogen (N) fixed determined by the N difference (ND) method and the ^{15}N natural abundance (NA) method for 2009 (A) and 2010 (B) sites using weeds (filled circles) or wheat (open circles) as the reference plant for the NA method.

References

Androsoff GL, Kessel C, Pennock DJ (1995) Landscape-scale estimates of dinitrogen fixation by *Pisum sativum* by nitrogen-15 natural abundance and enriched isotope dilution. Biol Fert Soils 20 (1):33-40.

Biederbeck VO, Bouman OT, Campbell CA, Bailey LD, Winkleman GE (1996) Nitrogen benefits from four green-manure legumes in dryland cropping systems. Can J Plant Sci 76 (2):307-315.

Boddey RM, Peoples MB, Palmer B, Dart PJ (2000) Use of the N-15 natural abundance technique to quantify biological nitrogen fixation by woody perennials. Nutr Cycl Agroecosys 57 (3):235-270.

Brandt SA (1996) Alternatives to summerfallow and subsequent wheat and barley yield on a dark brown soil. Can J Plant Sci 76 (2):223-228.

Bremer E, Gehlen H, Swerhone GDW, Vankessel C (1993) Assessment of reference crops for the quantification of N_2 fixation using natural and enriched levels of N-15 abundance. Soil Biol Biochem 25 (9):1197-1202.

Bremer E, Rennie RJ, Rennie DA (1988) Dinitrogen fixation of lentil, field pea and fababean under dryland conditions. Can J Soil Sci 68 (3):553-562.

Bundy LGM, J.J. (1994) Nitrogen availability indices., vol SSSA Book Ser. 5. Methods of Soil Analysis. Part 2. Biological Methods. Soil Science Society of America, Madison, WI.

Castellanos JZ, Peña-Cabriales JJ, Acosta-Gallegos JA (1996) 15N-determined dinitrogen fixation capacity of common bean (*Phaseolus vulgaris*) cultivars under water stress. J Agric Sci 126 (03):327-333.

Chen C, Miller P, Muehlbauer F, Neill KE, Wichman D, McPhee K (2006) Winter pea and lentil response to seeding date and micro- and macro-environments. Agron J 98 (6):1655-1663.

Dakora FD, Spriggs AC, Ndakidemi PA, Belane A (2008) Measuring N_2 fixation in legumes using ^{15}N natural abundance: some methodological problems associated with choice of reference plants. In: Dakora FD, Chimphango SBM, Valentine AJ, Elmerich C, Newton WE (eds) Biological Nitrogen Fixation: Towards Poverty Alleviation through Sustainable Agriculture, vol 42. Current Plant Science and Biotechnology in Agriculture. Springer, Dordecht, The Netherlands, pp 43-45.

Danso SKA (1995) Assessment of biological nitrogen fixation. Fert Res 42 (1-3):33-41.

Gan YT, Johnston AM, Knight JD, McDonald C, Stevenson C (2010) Nitrogen dynamics of chickpea: Effects of cultivar choice, N fertilization, Rhizobium inoculation, and cropping systems. Can J Plant Sci 90 (5):655-666.

Hardarson G, Danso SKA (1993) Methods for measuring biological nitrogen-fixation in grain legumes. Plant Soil 152 (1):19-23.

Hauggaard-Nielsen H, Holdensen L, Wulfsohn D, Jensen ES (2010) Spatial variation of N-2-fixation in field pea (*Pisum sativum* L.) at the field scale determined by the N-15 natural abundance method. Plant Soil 327 (1-2):167-184.

Houngnandan P, Yemadje RGH, Oikeh SO, Djidohokpin CF, Boeckx P, Van Cleemput O (2008) Improved estimation of biological nitrogen fixation of soybean cultivars (Glycine max L. Merril) using ^{15}N natural abundance technique. Biol Fert Soils 45 (2):175-183.

Izard EJ (2007) Seeking sustainability for organic cropping systems in the northern Great Plains: Legume green manure management strategies. Montana State University, Bozeman, MT

Jensen ES (1987) Seasonal patterns of growth and nitrogen-fixation in field-grown pea. Plant Soil 101 (1):29-37.

Kirda C, Danso SKA, Zapata F (1989) Temporal water-stress effects on nodulation, nitrogen accumulation and growth of soybean. Plant Soil 120 (1):49-55.

Kirkegaard J, Christen O, Krupinsky J, Layzell D (2008) Break crop benefits in temperate wheat production. Field Crops Res 107 (3):185-195.

Larney FJ, Lindwall CW, Bullock MS (1994) Fallow management and overwinter effects on wind erodibility in southern Alberta. Soil Sci Soc Am J 58 (6):1788-1794.

Miller PR, Engel RE, Holmes JA (2006) Cropping sequence effect of pea and pea management on spring wheat in the northern Great Plains. Agron J 98 (6):1610-1619.

Miller PR, Lighthiser EJ, Jones CA, Holmes JA, Rick TL, Wraith JM (2011) Pea green manure management affects organic winter wheat yield and quality in semiarid Montana. Can J Plant Sci 91 (3):497-508.

Nyemba RC, Dakora FD (2005) Identifying suitable arable weeds as reference plants for measuring N-2 fixation in grain legumes in Zambia using N-15 natural abundance. Symbiosis 40 (2):79-86.

Pate JS, Unkovich MJ, Armstrong EL, Sanford P (1994) Selection of reference plants for N-15 natural-abundance assessment of N-2 fixation by crop and pasture legumes in south-west Australia. Aust J Agric Res 45 (1):133-147.

Peoples MB, Boddey RM, Herridge DF (2002) Quantification of nitrogen fixation. In: Leigh GJ (ed) Nitrogen Fixation at the Millennium. Elsevier Science, Amsterdam, pp 357-389.

Rennie RJ (1984) Comparison of N balance and N-15 isotope-dilution to quantify N-2 fixation in field-grown legumes. Agron J 76 (5):785-790.

Rennie RJ, Dubetz S (1986) N-15 - determined nitrogen-fixation in field-grown chickpea, lentil, fababean, and field pea. Agron J 78 (4):654-660.

Salon C, Munier-Jolain NG, Duc G, Voisin AS, Grandgirard D, Larmure A, Emery RJN, Ney B (2001) Grain legume seed filling in relation to nitrogen acquisition: A review and prospects with particular reference to pea. Agronomie 21 (6-7):539-552.

Serraj R, Sinclair TR, Purcell LC (1999) Symbiotic N-2 fixation response to drought. J Exp Bot 50 (331):143-155.

Shearer G, Kohl DH (1986) N-2 fixation in field settings - estimations based on natural N-15 abundance. Aust J Plant Physiol 13 (6):699-756.

Thomas, Robertson MJ, Fukai S, Peoples MB (2004) The effect of timing and severity of water deficit on growth, development, yield accumulation and nitrogen fixation of mungbean. Field Crops Res 86 (1):67-80.

Unkovich M, Herridge DF, Peoples MB, Cadisch G, Boddey RM, Giller KE, Alves B, Chalk PM (2008) Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR Monograph. Australian Centre for International Agricultural Research, Canberra, AU.

Unkovich MJ, Baldock J, Peoples MB (2010) Prospects and problems of simple linear models for estimating symbiotic N-2 fixation by crop and pasture legumes. Plant Soil 329 (1-2):75-89.

Unkovich MJ, Pate JS (2000) An appraisal of recent field measurements of symbiotic N-2 fixation by annual legumes. Field Crops Res 65 (2-3):211-228.

Unkovich MJ, Pate JS, Sanford P, Armstrong EL (1994) Potential precision of the delta-N-15 natural-abundance method in-field estimates of nitrogen-fixation by crop and pasture legumes in south-west Australia. Aust J Agric Res 45 (1):119-132.

Walley FL, Fu GM, van Groenigen JW, van Kessel C (2001) Short-range spatial variability of nitrogen fixation by field-grown chickpea. Soil Sci Soc Am J 65 (6):1717-1722.

Zentner RP, Campbell CA, Biederbeck VO, Miller PR, Selles F, Fernandez MR (2001) In search of a sustainable cropping system for the semiarid Canadian prairies. J Sustainable Agric 18 (2-3):117-136.

Zentner RP, Campbell CA, Biederbeck VO, Selles F, Lemke R, Jefferson PG, Gan Y (2004) Long-term assessment of management of an annual legume green manure crop for fallow replacement in the Brown soil zone. Can J Plant Sci 84 (1):11-22.

Zentner RP, Campbell CA, Selles F, Jefferson PG, Lemke R, McConkey BG, Fernandez MR, Hamel C, Gan Y, Thomas AG (2006) Effect of fallow frequency, flexible rotations, legume green manure, and wheat class on the economics of wheat production in the Brown soil zone. Can J Plant Sci 86 (2):413-423.

CHAPTER 3

GROWTH, NUTRIENT UPTAKE, AND N FIXATION OF TWO LEGUME CROPS TO PHOSPHORUS FERTILIZATION AND INOCULATION WITH ARBUSCULAR MYCORRHIZAE

Introduction

Legume crops often have a higher requirement for phosphorus (P) than non-N fixing crops due to their nitrogen (N) fixation symbiosis (Li et al. 2011). In addition to the P necessary for the plant, the rhizobia symbiont also requires adequate levels of P to biologically fix $N_{2(g)}$ from the atmosphere. Biological N fixation is an energy- and P-intensive process, requiring approximately 16 mol of P-rich energy (ATP) for every 1 mol of N_2 fixed (Ruiz-Lozano et al. 2001). Additionally, P has a specific role in the formation and functioning of root nodules (O'Hara 2001), the organelles where N fixation takes place. Consequently, soil P deficiencies can limit legume crop yield and the amount of N fixed (Israel 1987). Many soils in the northern Great Plains cropping region test below critical soil P levels for major crops because of high soil calcium content (Fixen et al. 2011); however, many producers in the region often do not add any P during the legume rotation (Burgess et al. in review). Thus, we hypothesize that increasing P availability of low P soils in this region has the potential to positively affect both legume yield and the quantity of N fixed by the crop. Increasing legume N fixation can have valuable benefits for subsequent crops, especially when legumes are terminated as green manures.

Phosphorus fertilization is a simple and effective approach for improving P availability in conventional cropping systems. In soils with low to moderate P levels, P fertilization has been reported to increase biomass and shoot N and P uptake for a number of legume crops, yet the effects of P fertilization on legume N fixation are varied (Bremer et al. 1989; Cadisch et al. 1993; Walley et al. 2005). For example, a field study in Saskatchewan found P fertilization to increase lentil (*Lens culinaris*) biomass, but not N fixation, indicating the legume host was more responsive to P fertilization than the rhizobia (Bremer et al. 1989). Alternatively, Cadish et al. (1993) found P fertilization to increase yield, N uptake, and N fixation of two forage pea crops (*Centrosema acutifolium* and *C. macrocarpum*) in Australia.

For low-input conventional or organic systems, other options for increasing plant available P levels are of interest (Rick et al. 2011). Arbuscular mycorrhizae (AM) are a plant-fungal symbiosis that occur in a majority of terrestrial plants, including most legumes, resulting in a tripartite symbiosis with the legume host, AM fungi (AMF), and rhizobia. An important, well-established effect of the AM symbiosis on the host plant is an increase in P uptake, particularly when plant available P is limiting (Koide 1991). Fine AMF hyphae extend outside of the plant roots, thereby increasing the effective rooting zone and accessing P that is unavailable to the larger plant roots (Bolan 1991). Because of their role in improving P uptake, AMF may be particularly beneficial to legumes and specifically N fixation. Two mechanisms have been proposed for the role of AM in improving legume growth and N accumulation. First, AM can improve the health and vigor of the legume plant through increased P uptake, which can improve N fixation

through increased allocation of P and carbon from the legume host to the rhizobia. Second, AM can have a direct, localized effect on N fixation, possibly by stimulating nodule formation and function (Barea et al. 2005; Goss and de Varennes 2002). Chalk et al. (2006) report a number of studies in which both native and inoculated AMF treatments increased legume dry matter, grain yield, N and P uptake, and the percent of plant N that was fixed. Because most of these studies were conducted in sterile soils, the authors concluded that questions still remain regarding AM function in non-sterile conditions where agronomic and naturally-occurring conditions, such as presence of native AMF, can affect the symbiosis.

The adoption of AM into agricultural systems has been slow in the past and the effectiveness of the symbiosis in highly managed systems is not well understood (Hart and Trevors 2005; Ryan and Graham 2002). Agronomic practices, such as tillage, long fallow periods, certain crop rotations, or soils with high available P, can disrupt native AMF communities and reduce their infectivity potential over time (Jansa et al. 2006). A number of AMF inocula have been developed to improve AMF infectivity potential; however, the true root-infecting ability of these inocula are often unknown (Tarbell and Koske 2007). Furthermore, for soils with native AMF populations, the effect of commercial AMF inocula on plant response is unclear (Chalk et al. 2006; Ortas 2003).

The objective of this greenhouse study was to determine the effect of increased plant available P as affected by P fertilization and AMF inoculation on the growth and N fixation by spring pea (*Pisum sativum* cv. Arvika) and spring lentil (*Lens culinaris* cv. Richlea) in a soil with a native AMF community present. The study design was focused

on legumes grown as a green manure crop, though study implications were intended to be relevant for all types of legume production.

Materials and Methods

Experimental Design, Set-up, and Maintenance

The experiment was conducted in the Plant Growth Center at Montana State University-Bozeman from June-Aug. 2010 with three fixed factors: crop species (pea, lentil), P fertilization rates (0, 4, 8 mg P kg^{-1}), and AMF inoculum (AMF-, AMF+). Each treatment was replicated five times. A soil sterilization treatment was also part of this experiment; however, because of a large increase in soil nitrate levels as a result of steam sterilization, the sterilized soil treatment was analyzed and discussed separate of the non-sterilized soil experiment presented here (Appendix E).

Field soil (a frigid Typic Argiustoll; Williams loam) was collected in April 2010 to a depth of 0.15 m from an agricultural field under continuous durum wheat (*Triticum durum*) and dry pea (*Pisum sativum*) production since 2005 near Plentywood, MT ($48^{o}34'$ N, $104^{o}25'$ W). Soil was air-dried, flail-milled, sieved (<9.5 mm), and thoroughly mixed. A subsample of field soil was sieved to 2 mm and analyzed by an independent laboratory. Soil characteristics were as follows: pH, 8.0; organic carbon, 12 g kg^{-1}; available N, 8 mg kg^{-1}; P (Olsen), 10 mg kg^{-1}; K (ammonium acetate), 455 mg kg^{-1}; and sulfate-S, 7 mg kg^{-1}. Soil texture was comprised of 49% sand, 30% silt and 21% clay. The final potting soil was an unsterilzed 3:1 (v/v) mix of field soil and 0.8-2.0 mm (20/30 grit) quartz silica sand (Lane Mountain Co., Valley, WA).

Seeding occurred on 25 June by placing seeds into sterilized (10% bleach solution) plastic pots (15.2 cm x 15.2 cm deep) filled with 1.7 kg-soil:sand mix. Phosphorus fertilizer was applied prior to seeding by subsurface banding (3-cm depth) 10 mL of laboratory-grade $Na_2PO_4.H_2O$ at a rate of 0, 4, or 8 mg P kg-soil^{-1} down the midline of the pot. Laboratory-grade K_2SO_4 was applied with each P fertilizer solution at a rate of 5 mg S kg-soil^{-1}. Two pea and four lentil seeds were sown at a depth of 2 cm and placed 2 cm from the midline of each pot (1 cm above and 2 cm over from the banded fertilizer). Commercial granular rhizobia inoculum (Optimize Pulse IF for lentil and pea, EMD Crop Bioscience, Brookfield, WI) was applied directly below all seeds at a rate of 0.3 g kg-soil^{-1}. For the AMF+ treatments, commercial AMF inoculum (MycoApply Endo, Mycorrhizal Applications, Inc., Grants Pass, OR) was applied directly below the seeds at a rate of 0.44 g kg-soil $^{-1}$. The inoculum included four AMF species (*Glomus intraradices, G. mosseae, G. aggregatum,* and *G. etunicatum*) and a manufacturer's listing of 27,200 propagules kg^{-1}. Plants were thinned 10 d after planting (DAP) to one pea and two lentils per pot to best match field seeding rates.

Soil moisture was maintained near field capacity (0.18 to 0.20 g water g-soil^{-1}) throughout the experiment by weighing every 24 to 48 h with an electronic balance, and irrigating to the upper end of the soil water content target range. Greenhouse conditions were as follows: temperature range of 15-26^{o}C and a natural light cycle of approximately 14 h day/10 h night. Pots were randomly arranged 11 DAP and systematically rotated on a weekly basis thereafter.

Biomass Harvest and Analysis

Plant harvest began 53 DAP when more than 50% of pea and lentil plants had one full bloom. All replicates within treatment were harvested on the same day. Shoot biomass from each pot was dried (50^{o}C, 72 h), weighed, and ground (Udy mill, <0.5 mm). Tissue subsamples for P analysis were digested (concentrated nitric acid/30% hydrogen peroxide) and analyzed using inductively coupled plasma atomic emission spectrometry (ICP-AES). Tissue subsamples for total N and isotope-^{15}N natural abundance were combusted and analyzed using an elemental analyzer interfaced to an isotope ratio mass spectrometer (Stable Isotope Facility, UC Davis). The main root system from each pot was harvested and dried (50^{o}C, 24 h) prior to storing at room temperature. To assess AMF colonization, roots were rehydrated, cleared in 2.5% KOH (m/m) solution, stained with Trypan blue (modified method of Phillips and Hayman 1970) and quantified using the magnified-intersections method (McGonigle et al. 1990). The number of root intersections counted for each pot ranged from 140-162.

N Fixation

Nitrogen fixation was estimated with the ^{15}N natural abundance (NA) method (Shearer and Kohl 1986). The NA method relies on the ^{15}N natural abundance of plant available soil N based on a non-N fixing reference plant relative to atmosphere N_2. We used a non-nodulating pea isoline (*P. sativum* cv. Sparkle) as the reference plant. Establishment and management of the reference plants matched the N-fixing pea (one plant per pot and five replicate pots within a treatment combination). The proportion of N fixed from the atmosphere (%Ndfa) was calculated from the following:

$$\%\text{Ndfa} = \left(\frac{\delta^{15}\text{N}_{\text{reference}} - \delta^{15}\text{N}_{\text{legume}}}{\delta^{15}\text{N}_{\text{reference}} - B}\right) * 100$$

where δ^{15}N is the per thousand enrichment relative to atmosphere (0‰) and *B* value refers to the δ^{15}N of lentil (-0.71) and pea (-0.73) grown under N-free conditions in a previous sand-culture greenhouse experiment (Appendix B). These values are within the range of previously published *B* values reported for lentil and field pea cultivars (Unkovich et al. 2008). Total aboveground N fixed was calculated by multiplying biomass yield, tissue N concentration, and %Ndfa/100.

Statistical Analyses

All statistical analyses were performed using JMP 8.0 statistical software (SAS Institute, 2010). Analysis of variance (ANOVA) was performed to examine significant treatment differences and interactions. Treatment differences were further evaluated using planned orthogonal contrasts and Fisher's protected least significant difference (LSD) test. Plant species, P fertilizer, and AMF inoculation were considered fixed effects. The proportion of root colonized by AMF data were arcsine square root transformed before statistical analysis. Correlation analyses were performed using simple linear regression and Pearson's partial coefficient (R). All treatments were tested for significance at the $P<0.05$ level.

Results

AMF Colonization

Relatively high rates of AMF root colonization were observed for both pea and lentil at each P fertilizer and AMF inoculum treatment level (Fig. 3.1). Mean treatment colonization rates ranged from 58-87% for lentil and 65-88% for pea. For lentil, P fertilization had a negative effect on colonization frequency with the highest colonization occurring in the 0P treatment (P=0.01). There were no differences in colonization frequency between the 4P and 8P treatments or between AMF treatments. Mean AMF colonization rates in pea were similar among all treatments. None of the measured plant parameters were correlated with AMF colonization of lentil or pea. Colonization frequency was not calculated for the non-N-fixing reference plants; however, a subset of root samples was examined and the presence of AMF features were verified in the roots of reference plants from each treatment combination.

Biomass and Nutrient Uptake

There was a positive effect of P fertilization on shoot biomass for lentil and pea (Table 1). Biomass was 34% and 45% higher for lentil and pea, respectively, in 4P than in 0P, and 8P increased biomass by approximately 60% over the control for both crops. Inoculation did not affect biomass production of either species. Planned contrasts, however, found pea biomass at the 4P rate to be greater in the AMF+ compared to AMF- treatments and were similar to biomass at the 8P rate.

Shoot N and P uptake were positively affected by P fertilization for both species (Table 2). This response was largely explained by biomass production as fertilization had

either no response or a negative response on tissue N and P concentrations. There was no overall inoculation effect or interaction on any of the measured N and P parameters, though AMF+ in the 4P pea treatments increased mean N uptake compared to the AMF- treatment and was similar to N uptake at the 8P rate. There was no inoculation effect or fertilizer by inoculation interaction on P uptake for either species.

N Fixation by Lentil

A main assumption of ^{15}N natural abundance method is that the non-N-fixing reference plant is enriched in ^{15}N abundance relative to the N-fixing plant. Mean lentil $\delta^{15}N$ values were greater than mean reference plant $\delta^{15}N$ values in over 50% of the replicates, resulting in negative N fixation estimates. As a result, it was not possible to directly quantify N fixation by lentil. Nodules were not observed on reference plant roots, so it is not known why this unexpected outcome occurred.

A qualitative assessment of treatment effects on lentil N fixation was accomplished by comparing lentil $\delta^{15}N$ values among treatments (Table 2). Atmospheric $N_{2(g)}$ has a uniform $\delta^{15}N$ signature of 0‰ (Shearer and Kohl 1986) and in soils enriched in^{15}N abundance, it is assumed that treatments that improve N fixation will lower the legume tissue $\delta^{15}N$ content (Azcón et al. 1991). There was a significant P fertilizer effect on lentil $\delta^{15}N$ values. Lentil fertilized at the 4P and 8P rates had lower $\delta^{15}N$ values than the 0P treatment, suggesting P fertilization increased lentil N fixation. Mean lentil $\delta^{15}N$ content at the 4P rate was lower in the AMF+ treatment than in the AMF- treatment, but at the 8P rate there was no difference in $\delta^{15}N$ values between AMF treatments. Pea $\delta^{15}N$ values had a pattern similar to lentil $\delta^{15}N$ values across treatments.

N Fixation by Pea

Pea $\delta^{15}N$ values were consistently lower than reference plant $\delta^{15}N$ values and N fixation was quantified for pea using the NA method (Table 3). Phosphorus fertilization increased the amount of N fixed and the %Ndfa . Averaged across AMF treatments, P fertilization increased %Ndfa from 10% in the 0P treatments to 61% and 53% in the 4P and 8P treatments, respectively. Inoculation did not affect N fixation, yet there was a fertilizer by inoculation interaction. At the 4P rate, the amount of N fixed was 49% higher in the AMF+ treatment than AMF- treatment. In contrast, at the 8P rate, the quantity of N fixed was reduced by more than 50% in the AMF+ treatment than the AMF- treatment

Discussion

P Fertilization

Positive response to P fertilization has been observed for a number of legume species in the NGP region, particularly in soils with P concentrations below critical levels (Bremer et al. 1989; McKenzie et al. 2001; Walley et al. 2005). This was confirmed in our study in which there was a consistent positive response in biomass production and nutrient uptake to P fertilization by both lentil and pea. Averaged across AMF treatments, biomass and N and P uptake response increased with increasing P fertilizer rates. Additionally, P fertilization increased N fixation by pea and likely lentil. Yet, unlike biomass and nutrient uptake, there was no additional gain in N fixation by pea between the 4P and 8P treatments. This finding suggests P fertilization was important for improving N fixation, yet the rhizobia symbiont was not as responsive as the legume host

to further increases in P availability, at least up through flower stage. Allocation of P from the legume host to the rhizobia symbiont can differ as the crop develops (Sinclair and Vadez 2002), thus the effect of P fertilization on N fixation may be different at a later growth stage.

AMF Inoculation

The addition of AMF inoculum did not have an overall effect on measured responses for either legume species. There were no differences in colonization rates between the AMF- and AMF+ treatments, indicating the field soil had a robust native AMF community with high infectivity potential. Studies reporting a positive response to AMF inoculation in non-sterile soil are often conducted in soils with low native AMF potential (Ganry et al. 1985; Ortas 2003). The field soil sourced for this study had a long history of low P fertility and no-till management, two agronomic practices that can foster native AMF populations over time (Jansa et al. 2006). Thus, our findings suggest that in soils with a robust, native AMF population applying commercial AMF inoculum may not be warranted.

P Fertilization and AMF Inoculation Interaction

There is a well-established inverse relationship between soluble soil P availability and AMF colonization (Koide 1991; Smith 1992). In our study, an inverse relationship between P fertilization and AMF colonization was only significant for lentil. This increase in colonization was apparently of little biological significance, however, as it did not affect other growth parameters. High AMF colonization observed among all P

fertilizer treatments indicate increases in soluble P availability were not high enough to deter the infectivity potential of the native and/or inoculum AMF.

A number of field and greenhouse studies conducted in soils with low soil P availability have concluded that an appropriate addition of soluble P is required to optimize the response to AMF inoculation (Ganry et al. 1985; Jansa et al. 2006). This was confirmed in our study in which AMF+ did not affect legume response in the 0P treatments, but there was an AMF+ effect on pea biomass, N uptake, and N fixation in the 4P treatments. This finding suggests AMF+ improved uptake of fertilizer P rather than soil P and may be the result of both spatial and temporal aspects of the inoculum treatment. Specifically, inoculum was placed directly below the seed and early establishment of AMF hyphae near the top of the root system would have been in close proximity (within 2.5 cm) of the applied band of P fertilizer. Conversely, native AMF propagules existed throughout the soil and initial establishment of the symbiosis may have been delayed (Oliveria and Sanders 2000) and less concentrated near the primary roots, resulting in less fertilizer P uptake by native AMF. Early P uptake has been shown to be critical for later growth and development in a number of crops (Grant et al. 2001) and may improve early nodulation (O'Hara 2001), resulting in greater pea response to AMF inoculation. Nodule formation may have also been directly affected by faster AMF establishment in the AMF+ treatments. Goss and de Varennes (2002) found soybean roots with higher rates of AMF colonization at 10 d after emergence increased nodule numbers and nodule dry weight compared to soybean treatments with lower AMF colonization rates at the same stage, though P uptake was similar between the two

treatments. By pod-fill, AMF colonization rates and the number of nodules were the same between the two treatments, yet nodule dry weight and N fixation were significantly higher in the faster AMF-establishing treatments. Nodule parameters were not measured in the present study, but further research into early initiation of AMF colonization and nodule formation may shed light upon the AMF-by-rhizobia interaction.

An AMF+ benefit for pea was not observed in the 8P treatments. The lack of P uptake response when P was increased from 4P to 8P in both AMF treatments suggests pea was less dependent on P uptake by AMF, including inoculum AMF, at the high P rate (Kahiluoto et al. 2000). It is unclear why AMF+ reduced pea N fixation at the 8P rate. Establishment of AMF colonization in the AMF+ treatments may have been delayed due to high P availability near the seed (Jansa et al. 2011), though this does not account for higher N fixation in the AMF- treatment.

There was less of an AMF+ effect within P fertilizer treatments for lentil. A comparison of lentil $\delta^{15}N$ values at the 4P rate suggests N fixation may have been greater in the AMF+ than AMF- treatments, though there was not a significant increase in biomass or N uptake in the same treatments. Xavier and Germida (2002, 2003) found species-specific interactions among AMF, rhizobia, and plant host species to have varying effects on the growth and N fixation of lentil and pea in non-sterile soils. Positive effects on plant growth and N fixation were only observed when certain AMF species were matched with specific rhizobia bacteria and a plant host. The rhizobia and AMF species present in the roots of each host plant were not identified in this study and it is not known how the native microorganism populations may have interacted with the rhizobia

and AMF inoculum populations and the two legume hosts. Further research will be required to explore the effect of species-specific compatibility and their interaction with plant P availability within the tripartite symbiosis.

Conclusions

Phosphorus availability is known to increase N fixation of legumes, yet the synergistic effects of P fertilization and AMF inoculation are relatively unknown in non-sterilized soils. Phosphorus fertilization was most effective for improving plant response for lentil and pea in this study, even at moderate application rates. Inoculation with AMF did not have a direct effect on AMF colonization or legume performance, though there was evidence implying AMF+ improved pea growth and N fixation when a moderate addition of fertilizer P was applied. Overall, our findings indicate that in soils with a healthy native AMF population, inoculation with commercial AMF is likely not warranted. Future work is needed to further investigate the interaction between AMF inoculation and soil P availability in field conditions as well as species-specific interactions within the tripartite symbiosis.

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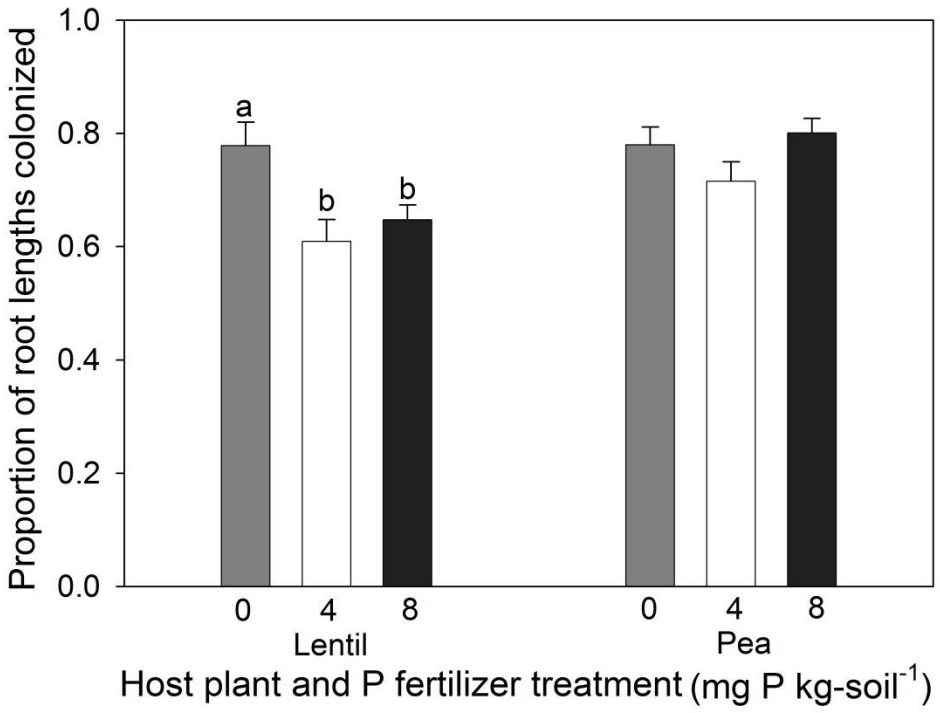

Figure 3.1. The proportion (mean + SE) of root intersections colonized by arbuscular mycorrhizae fungi (AMF) for lentil (A) and pea (B) at three phosphorus (P) fertilization rates (0, 4, and 8 mg P kg-soil^{-1}). Bars with the same letter within a species are not statistically different ($P<0.05$). Colonization levels were the same between AMF treatments and data were pooled over AMF treatments.

Table 3.1. Biomass, shoot nitrogen (N) and phosphorus (P) uptake, and tissue N and P concentrations for lentil and pea under three P fertilization rates (mg P kg-soil^{-1}) without (-) or with (+) arbuscular mycorrhizae fungi (AMF) inoculum. Columns with the same letter are not statistically different (P<0.05).

P fertilizer rate	AMF	Biomass (g pot^{-1})	N uptake (mg pot^{-1})	P uptake (mg pot^{-1})	Tissue N (g kg^{-1})	Tissue P (g kg^{-1})
			Lentil			
0	-	1.24c	35.2c	3.3c	28.5	2.7
	+	1.21c	34.5c	3.3c	29.1	2.7
4	-	1.62b	39.2bc	4.1b	24.3	2.6
	+	1.68b	45.8ab	4.4b	27.2	2.6
8	-	2.01a	51.9a	5.3a	26.0	2.6
	+	1.95a	52.1a	5.2a	26.8	2.7
$LSD_{0.05}$		*0.25*	*6.7*	*0.6*	*n.s.*	*n.s*
Source of variation	*df*			*P values*		
P fertilizer (P)	2	<0.01	<0.01	<0.01	0.11	0.59
AMF inoculation (I)	1	0.84	0.33	0.72	0.22	0.48
P x I	2	0.85	0.92	0.93	0.94	0.91
			Pea			
0	-	2.02c	57.7c	4.5c	28.5	2.2ab
	+	1.97c	59.8c	4.6bc	30.5	2.4a
4	-	2.66b	76.8b	5.3ab	29.2	2.0bc
	+	3.11a	90.9a	5.0abc	29.0	1.6d
8	-	3.20a	88.4ab	5.5a	27.7	1.7d
	+	3.14a	81.3ab	5.8a	25.9	1.8cd
$LSD_{0.05}$		*0.41*	*13.5*	*0.8*	*n.s.*	*0.3*
Source of variation	*df*			*P values*		
P fertilizer (P)	2	<0.01	<0.01	<0.01	0.04	<0.01
AMF inoculation (I)	1	0.46	0.50	0.91	0.97	0.63
P x I	2	0.95	0.42	0.94	0.15	0.74

Table 3.2. Mean tissue $\delta^{15}N$ content for lentil and pea across three P fertilization rates without (-) or with (+) arbuscular mycorrhizae fungi (AMF) inoculum. Columns with the same letter are not statistically different ($P<0.05$).

P fertilizer rate (mg P kg-soil^{-1})	AMF	$\delta^{15}N$ (‰)	
		Lentil	Pea
0	-	1.96ab	0.85b
	+	2.32a	1.42a
4	-	1.89b	0.41bc
	+	1.09c	-0.19d
8	-	1.09c	-0.01cd
	+	1.02c	0.08cd
$LSD_{0.05}$		*0.43*	*0.46*
Source of variation	*df*	*P values*	
P fertilizer (P)	2	<0.01	<0.01
AMF inoculation (I)	1	0.25	0.90
P x I	2	0.23	0.28

Table 3.3. Mean amounts of nitrogen (N) fixed and the percentage of N derived from the atmosphere (%Ndfa) by pea across three phosphorus (P) fertilization rates without (-) or with (+) arbuscular mycorrhizae fungi (AMF) inoculum. Columns with the same letter are not statistically different (P<0.05).

P fertilizer rate (mg P kg-soil^{-1})	AMF	N fixed (mg pot^{-1})	%Ndfa
0	-	1.3d	0.0e
	+	12.7cd	19.8de
4	-	38.3b	48.3bc
	+	67.7a	73.9a
8	-	61.1a	68.3ab
	+	29.7bc	37.3cd
$LSD_{0.05}$		*21.9*	*25.4*
Source of variation	*df*	*P values*	
P fertilizer (P)	2	<0.01	<0.01
AMF inoculation (I)	1	0.71	0.60
P x I	2	0.05	0.03

References

Azcón R, Rubio R, Barea JM (1991) Selective interactions between different species of mycorrhizal fungi and *Rhizobium meliloti* strains, and their effects on growth, N2-fixation (15N) and nutrition of *Medicago sativa* L. New Phytol 117 (3):399-404.

Barea JM, Werner D, Azcón-Aguilar C, Azcón R (2005) Interactions of arbuscular mycorrhiza and nitrogen-fixing symbiosis in sustainable agriculture. In: Werner D, Newton WE (eds) Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment, vol 4. Springer, Dordrecht, The Netherlands, pp 199-222.

Bolan NS (1991) A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. Plant Soil 134 (2):189-207.

Bremer E, Vankessel C, Karamanos R (1989) Inoculant, phosphorus and nitrogen responses of lentil. Can J Plant Sci 69 (3):691-701.

Burgess M, Miller PR, Jones CA (in review) Pulse crops improve energy intensity and productivity of cereal production in Montana, USA. J Sustainable Agric.

Cadisch G, Sylvester-Bradley R, Boller BC, Nosberger J (1993) Effects of phosphorus and potassium on N-2 Fixation (N-15-Dilution) of field-grown *Centrosema acutifolium* and *C. macrocarpum* Field Crops Res 31 (3-4):329-340.

Chalk PM, Souza RD, Urquiaga S, Alves BJR, Boddey RM (2006) The role of arbuscular mycorrhiza in legume symbiotic performance. Soil Biol Biochem 38 (9):2944-2951.

Fixen PE, Bruulsema TW, Jensen TL, Mikkelsen R, Murrell TS, Phillips SB, Rund Q, Stewart WM (2011) The fertility of North American soils, 2010. Better Crops, vol 94.

Ganry F, Diem HG, Wey J, Dommergues YR (1985) Inoculation with *Glomus mosseae* improves N-2 fixation by field-grown soybeans. Biol Fert Soils 1 (1):15-23.

Goss MJ, de Varennes A (2002) Soil disturbance reduces the efficacy of mycorrhizal associations for early soybean growth and N-2 fixation. Soil Biol Biochem 34 (8):1167-1173.

Grant CA, Flaten DN, Tomasiewicz DJ, Sheppard SC (2001) The importance of early season phosphorus nutrition. Can J Plant Sci 81 (2):211-224.

Hart MM, Trevors JT (2005) Microbe management: application of mycorrhyzal fungi in sustainable agriculture. Front Ecol Environ 3 (10):533-539.

Israel DW (1987) Investigation of the role of phosphorus in symbiotic dinitrogen fixation. Plant Physiol 84 (3):835-840.

Jansa J, Finlay R, Wallander H, Smith FA, Smith SE (2011) Role of mycorrhizal symbioses in phosphorus cycling. In: Phosphorus in Action, vol 100. Soil Biology. Springer Berlin Heidelberg, pp 137-168.

Jansa J, Wiemken A, Frossard E (2006) The effects of agricultural practices on arbuscular mycorrhizal fungi. Geological Society, London, Special Publications 266 (1):89-115.

Kahiluoto H, Ketoja E, Vestberg M (2000) Promotion of utilization of arbuscular mycorrhiza through reduced P fertilization 1. Bioassays in a growth chamber. Plant Soil 227 (1):191-206.

Koide RT (1991) Nutrient supply, nutrient demand and plant-response to mycorrhizal infection New Phytol 117 (3):365-386.

Li SX, Wang ZH, Stewart BA (2011) Differences of some leguminous and nonleguminous crops in utilization of soil phosphorus and responses to phosphate fertilizers. In: Advances in Agronomy, vol 110. pp 125-249.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A new method which gives an objective-measure of colonization of roots by vesicular arbuscular mycorrhizal fungi. New Phytol 115 (3):495-501.

McKenzie RH, Middleton AB, Solberg ED, DeMulder J, Flore N, Clayton GW, Bremer E (2001) Response of pea to rate and placement of triple superphosphate fertilizer in Alberta. Can J Plant Sci 81 (4):645-649.

O'Hara GW (2001) Nutritional constraints on root nodule bacteria affecting symbiotic nitrogen fixation: a review. Aust J Exp Agr 41 (3):417-433.

Oliveira AAR, Sanders FE (2000) Effect of inoculum placement of indigenous and introduced arbuscular mycorrhizal fungi on mycorrhizal infection, growth, and dry matter in Phaseolus vulgaris. Trop Agric 77 (4):220-225.

Ortas I (2003) Effect of selected mycorrhizal inoculation on phosphorus sustainability in sterile and non-sterile soils in the Harran Plain in South Anatolia. J Plant Nutr 26 (1):1-17.

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:157-160.

Rick T, Jones C, Engel R, Miller P (2011) Green manure and phosphate rock effects on phosphorus availability in a northern Great Plains dryland organic cropping system. Org Agr 1 (2):81-90.

Ruiz-Lozano JM, Collados C, Barea JM, Azcon R (2001) Arbuscular mycorrhizal symbiosis can alleviate drought-induced nodule senescence in soybean plants. New Phytol 151 (2):493-502.

Ryan MH, Graham JH (2002) Is there a role for arbuscular mycorrhizal fungi in production agriculture? Plant Soil 244 (1-2):263-271.

Shearer G, Kohl DH (1986) N-2 fixation in field settings - estimations based on natural N-15 abundance. Aust J Plant Physiol 13 (6):699-756.

Sinclair TR, Vadez V (2002) Physiological traits for crop yield improvement in low N and P environments. Plant Soil 245 (1):1-15.

Smith VH (1992) Effects of nitrogen: Phosphorus supply ratios on nitrogen fixation in agricultural and pastoral ecosystems. Biogeochemistry 18 (1):19-35.

Tarbell TJ, Koske RE (2007) Evaluation of commercial arbuscular mycorrhizal inocula in a sand/peat medium. Mycorrhiza 18 (1):51-56.

Unkovich M, Herridge DF, Peoples MB, Cadisch G, Boddey RM, Giller KE, Alves B, Chalk PM (2008) Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR Monograph. Australian Centre for International Agricultural Research, Canberra, AU.

Walley FL, Kyei-Boahen S, Hnatowich G, Stevenson C (2005) Nitrogen and phosphorus fertility management for desi and kabuli chickpea. Can J Plant Sci 85 (1):73-79.

Xavier LJC, Germida JJ (2002) Response of lentil under controlled conditions to co-inoculation with arbuscular mycorrhizal fungi and rhizobia varying in efficacy. Soil Biol Biochem 34 (2):181-188.

Xavier LJC, Germida JJ (2003) Selective interactions between arbuscular mycorrhizal fungi and *Rhizobium leguminosarum* bv. viceae enhance pea yield and nutrition. Biol Fert Soils 37 (5):261-267.

CHAPTER 4

SUMMARY

Two main studies were conducted to better understand nitrogen (N) fixation by annual legumes in semi-arid cropping systems of the northern Great Plains (NGP), with a special focus on legume green manures. The first was a two-year field study and the second was a greenhouse study.

The field study evaluated the effect of planting time and termination time on N fixation by field pea and lentil. This study found that when growing season precipitation was above normal, terminating at pod stage compared to flower stage increased the quantity of N fixed; however, when growing season precipitation was below normal, there was no additional N fixed by pea terminated at pod than flower. Lentil continued to fix N through pod stage in both years. Although this study was conducted in a conventional cropping system, findings from this study can have implications for organic farmers utilizing LGM crops as well.

Other than N, soil nutrient levels were optimized in the field study; however soil nutrient deficiencies, particularly phosphorus (P) deficiencies, can limit both legume yields and the quantity of N fixed. Because many soils in the NGP are low in soil available P, this led me to conduct a greenhouse experiment to investigate the effect of increasing soil phosphorus (P) availability through P fertilization and arbuscular mycorrhizae fungus (AMF) inoculation on lentil and pea growth and N fixation. The study determined that there was a positive P fertilization effect on biomass and N and P

uptake for both legumes and an N fixation benefit for pea. Inoculation with AMF did not affect measured parameters; however, there was evidence that AMF inoculation improved pea performance when P availability was moderate. Results from this study suggest that improving P availability in soils with low P levels can be warranted given an increase in legume yields and the amount of N fixed. Future work is needed to investigate the role AMF and its interaction with N fixation processes and soil P levels may play in legume cropping systems in the NGP.

In conclusion, this research was conducted to assess how management decisions and soil fertility conditions impact legume N fixation. To my knowledge, N fixation by LGMs had not been directly estimated in Montana and findings from this project should be useful for evaluating the potential contribution of fixed N to an agroecosystem as well as identifying soil and environmental factors that can affect N fixation in semi-arid cropping regions. Follow-up research to this work would be further investigation into the cycling of fixed N in agroecosystems, including the effect of LGMs on subsequent non-legume crops and long-term influences on soil N and carbon content. Continuing research into the mechanisms involved with N fixation and its interaction with other microbial communities, such as AMF, will offer further insight to the role of biological N fixation in sustainable cropping systems.

APPENDICES

APPENDIX A

SOIL NITROGEN AND CARBON CONTENT IN TWO LONG-TERM CROP ROTATION STUDIES

Introduction

The northern Great Plains (NGP) cropping region has historically been dominated by cereal crop-fallow management; however, there has been a steady increase in more diversified crop rotations systems, including legume-based rotations, in recent years (Grant et al. 2002). Legume-based cropping systems may improve soil N and C content over time as a result of increased N inputs to the system through N fixation and incorporation of high-quality leguminous residues. Yet, estimates of the long-term contribution of legume crops to NGP agroecosystems vary widely (Walley et al. 2007). The purpose of Appendix A is to evaluate the effect of various legume-based rotations on soil total N and total C content as compared to traditional wheat-based systems and rotations without legumes in two long-term crop rotation studies near Bozeman, Montana.

Materials and Methods

Experimental Design

Crop Diversification Rotation Study The crop diversification rotation study (CDRS) was initiated in 2000 at the Montana State University Arthur Post Farm approximately 10 km west of Bozeman, MT (45°40'N, 111°09′ W). A description of the study site and details of the design and methods for the first four years of this experiment have been reported previously (Miller et al. 2008). Therefore, only a brief review is presented here, including management changes for the latter four years. The 2000-2003 design included four no-till cropping systems and one organic cropping system and was

fully-phased (Table A.1.). In 2004, the study was converted to dual-phase to minimize pest problems and the following crop rotation treatments were added: mustard was added to the winter (NTW) and spring (NTS) third-year phase, an additional corn rotation was added to the diverse (NTD) rotation, and a 'natural' pesticide-free production (PFP) rotation was included as a counterpart to the organic rotation in which soil residual and in-crop pesticides were avoided, yet N was applied at the recommended rate (Fig. A.1., Table A.2.). Winter triticale replaced winter wheat as the last year phase crop in both NTW rotations. Each of the rotations, except for organic, natural, and continuous wheat, had both a recommended (H) and half recommended (L) N treatment (whole plot level). Recommended N rates per 100 kg of targeted yield were 3, 5, 6, and 6.5 kg N for corn, wheat, safflower, and canola/flax, respectively. The experimental design was a randomized complete block with four replicates, and crop system phases were randomized to plots within each block. Plot size was 7.3 by 14.6 m.

GGRS The greenhouse gas rotation study (GGRS) was initiated in 2003 and is adjacent to the CDRS study. A description of the study site and some of the design and methods of the experiment have been previously discussed (Dusenbury et al. 2008). Thus, only a brief description of the study design as it pertains to this study is presented. The experimental design was established with eight cropping systems (Fig. A.2.; Table A.3.). All but the continuous wheat (CW) rotation consisted of two phases and crop rotations were single-phased. Starting in 2004, the experimental design was a split-plot RCBD with crop rotation as the main plot effect and N fertilizer treatment as the subplot effect, excluding the organic rotation which did not receive any N fertilizer. Nitrogen

fertilizer treatments represented low (L; available N=100 kg ha^{-1}) and high (H; available N=200 kg ha^{-1}) N fertility based on routine soil tests. Fertilizer was applied only in the wheat rotation year. Fertilizer rates were randomized in plots to either the north or south side of the plot. Plot size was 7.6 by 21.3 m.

Soil Sampling and Laboratory Analysis

Soil was sampled 2-18 Apr. 2008 at the CDRS site and 4-12 Sept. 2008 for the GGRS site. Soil sampling in Apr. 2008 was extended over numerous sampling days due to wet soil conditions. For both studies, soil was sampled to a depth of 0.6 m using a 30.1-mm diam. hydraulic soil probe. At the CDRS site, soil was sampled in each of four quadrants in each plot, generally 0.5 to 1 m from the edge of the plot. Soil cores were separated into three depths (0 to 15, 15 to 30, and 30 to 60-cm) and composited by depth. At the GGRS site, soil was sampled in two locations (east [A] and west [B]) within each subplot for the 0 to 30-cm samples. For the 30 to 60-cm depth samples, soil was sampled on the east (A)-side of the subplot for blocks 1 and 3 and the west (B)-side of the subplot for blocks 2 and 4. Three soil cores per location were collected, separated into four depths (0 to 10, 10 to 20, 20 to 30, and 30 to 60-cm), and composited by depth for that location.

For both studies, soil was collected in plastic-lined paper bags and stored in a cooler for transport from the field to laboratory. In the laboratory, a soil subsample of 20 g (wet weight) from each sample was dried (105^{o}C for 24 h) and weighed to determine bulk density and gravimetric water content. The remaining soil was dried (70^{o}C for 72 h), ground, and sieved (<2 mm). Prior to analysis, soil was re-dried (70^{o}C, overnight) and a

2.0 g subsample was analyzed for TN and TC with an automatic combustion analyzer (TruSpec CN, LECO Corp. St. Joseph, MI).

For CDRS, calculated soil bulk densities were abnormally low, likely as a result of the spring sampling time. Thus, bulk densities obtained from soil sampling in fall 2009 were used (1.39 g cm^{-3} for 0 to 30-cm depth; 1.33 g cm^{-3} for 30 to 60-cm depth). For GGRS, individual plot bulk density values were used. Plots with a bulk density below 1.2 g cm^{-3} or above 1.5 g cm^{-3} were removed as outliers and an average bulk density from other locations in the same plot was calculated for the removed value. Soil TN and TC content (kg ha^{-1}) were calculated for each depth from nutrient concentrations (g kg^{-1}), bulk densities, and length of soil core.

Statistical Analysis

All analyses were accomplished with JMP 8.0 statistical software (SAS Institute 2010). Analysis of variance (ANOVA) was performed to determine significant differences among treatments. Differences were further examined using planned orthogonal contrasts and Fisher's protected least significant difference (LSD) test. For CDRS, crop rotation plus fertilizer rate (crop system) was considered a main plot effect and year phases were combined across crop systems. For GGRS, N fertilizer rate was nested within crop system. Block was considered a random variable and crop system was a fixed variable. Missing data were omitted from individual soil depth analyses.

Results

CDRS

There was no difference in mean TN or TC among rotation treatments for any of the soil depths, including the combined 0 to 30 and 0 to 60-cm depths (Tables A.4., A.5.). *A priori* contrasts among the three rotations with an L and H fertilizer rate found a significant difference in TC in the top 0 to 15-cm depth for the NTD ($P<0.04$) and NTS ($P<0.04$) rotations. In both cases, average TC content was higher in the L fertilizer rate treatments than the H fertilizer rate treatments. This may have been the result of increased N and soil organic matter mineralization in the higher N fertilizer rate (H) treatments.

GGRS

Crop system had a significant effect on soil TN in each depth, except 30 to 60-cm, and the combined 0 to 30-cm depth (Table A.6). There was not a significant N fertilizer nested within crop system effect. Mean soil TN values (0 to 30-cm) by fertilizer rate (low and high) for each crop system are displayed in Table A.7. With the exception of SP-WW, all two-phase crop rotations and CW had higher TN in the 0 to 30-cm depth than the two fallow-wheat rotations. *A priori* contrasts found continuous wheat and legume-based systems (rotations 4, 5, 6, and 7) to have higher TN (0 to 30-cm) than fallow-wheat (rotations 1 and 2; $P<0.001$). There was no difference in TN (0 to 30-cm) between pea for grain (rotations 4 and 5) and pea for forage (rotations 6 and 7). There

were no differences in TC among crop systems or fertilizer rate nested within crop systems.

Conclusions

Findings from the analysis of soil TN and TC in two long-term studies reflect previous findings from NGP studies: contributions by legumes to soil N and C content vary (Walley et al. 2007). There were no differences in TN and TC among crop rotations in the CDRS, though cropping system did affect TN in the GGRS study with the two wheat-fallow systems and spring pea-winter wheat rotation consistently having the lowest amounts of TN in the 0 to 30-cm depth. There was no overall effect of fertilizer rate on soil TN or TC (0-30 or 0-60 cm soil depth) for either study. This may partly be the result of increased N removal in the higher fertilizer rate treatments, but the scale of N inputs relative to the much greater quantity of TN already in the system is also likely a factor. Thus, to fully assess the effect of legume-based crop rotations on soil N and C economies over time, a continuation of long-term studies that account for N inputs from N fixation and fertilizer and outputs from N and biomass harvested for grain or forage along with baseline and on-going soil tests are needed.

Replicate 4																
PFP3	**NTD3-H**	**PFP1**	**NTD1-L**	**NTS1-H**	**NTW1-H**	**NTS3-H**	**NTW3-L**	**NTS3-L**	**NTD1-H**	**NTS1-L**	**NTD3-L**	**CW1**	**NTW3-H**	**Org3**	**Org1**	**NTW1-L**
401 Lentil	402 Sunflwr	403 AWP	404 S Pea	405 S Pea	406 AWP	407 Canola	408 WCan	409 Mustard	410 S Pea	411 S Pea	412 Sunflwr	413 SW	414 WCan RR	415 Lentil	416 AWP Fallow	417 AWP
Replicate 3																
Org3	**NTW1-H**	**NTS1-H**	**PFP3**	**NTD3-L**	**PFP1**	**NTS3-H**	**Org1**	**NTW3-H**	**NTD1-L**	**NTW3-L**	**NTW1-L**	**NTD3-H**	**NTD1-H**	**NTS3-L**	**CW1**	**NTS1-L**
301 Lentil	302 AWP	303 S Pea	304 Lentil	305 Sunflwr	306 AWP	307 Canola	308 AWP Fallow	309 WCan RR	310 S Pea	311 WCan	312 AWP	313 Sunflwr	314 S Pea	315 Mustard	316 SW	317 S Pea
Replicate 2																
NTS3-H	**NTW3-L**	**NTD3-L**	**NTS3-L**	**Org3**	**PFP3**	**NTS1-H**	**NTS1-L**	**CW1**	**NTD3-H**	**NTW3-H**	**NTD1-H**	**NTW1-H**	**PFP1**	**NTD1-L**	**NTW1-L**	**Org1**
201 Canola	202 WCan	203 Sunflwr	204 Mustard	205 Lentil	206 Lentil	207 S Pea	208 S Pea	209 SW	210 Sunflwr	211 WCan RR	212 S Pea	213 AWP	214 AWP	215 S Pea	216 AWP	217 AWP Fallow
Replicate 1																
NTS3-L	**Org3**	**NTW1-L**	**NTS3-H**	**NTW3-L**	**Org1**	**CW1**	**NTD3-H**	**PFP3**	**NTS1-H**	**NTW3-H**	**NTD1-H**	**NTD3-L**	**NTW1-H**	**PFP1**	**NTS1-L**	**NTD1-L**
101 Mustard	102 Lentil	103 AWP	104 Canola	105 WCan	106 AWP Fallow	107 SW	108 Sunflwr	109 Lentil	110 S Pea	111 WCan RR	112 S Pea	113 Sunflwr	114 AWP	115 AWP	116 S Pea	117 S Pea

Figure A.1. Study map of the crop diversification rotation study. Rotation phase is for 2006/2007 crop year (odd-yr phase).

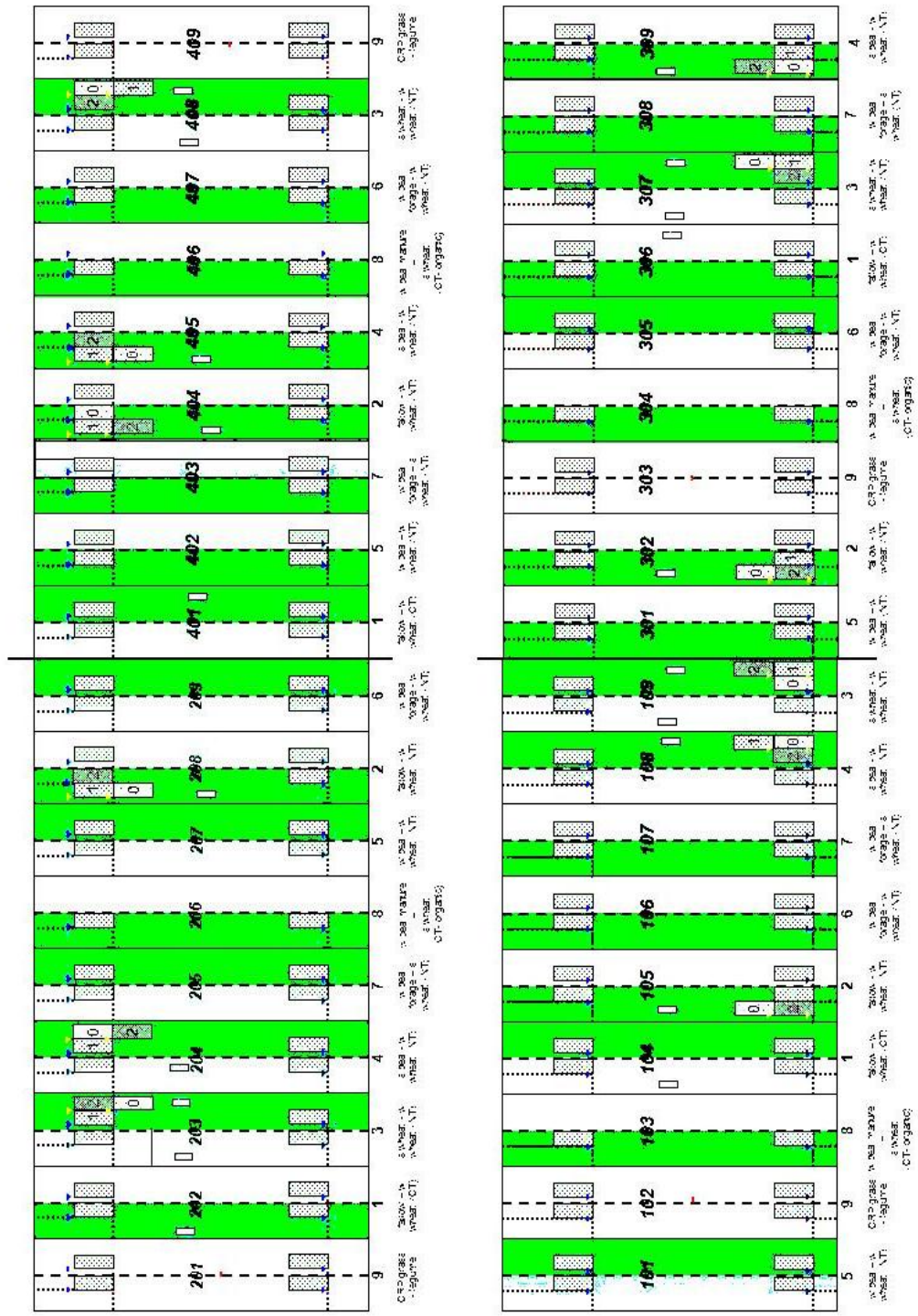

Figure A.2. Study map of the greenhouse gas rotation study, 2004-2007. The darkened half of a plot received the high fertilizer rate. Small boxes within a plot were for another component of this study and not discussed.

Table A.1. Planned phase sequence for Crop Diversification Rotation Study (CDRS) from 2000-2003 (Miller et al. 2008).

Crop System[a]	Rotation
1. ORG	(1) AWP manure - (2) W Wheat - (3) S Lentil - (4) Barley
2. NTW	(1) Winter Pea - (2) W Wheat - (3) Dormant S Canola - (4) W Wheat
3. NTS	(1) S Pea - (2) S Wheat - (3) Canola - (4) S Wheat
4. NTD	(1) S Pea - (2) W Wheat - (3) Hybrid Corn- (4) Sunflower
5. NTCW	(1) S Wheat- (2) W Wheat

[a] ORG=organic, NT=no-till, W=winter, S=spring, D=diverse, CW=continuous wheat, AWP=Austrian winter pea.

Table A.2. Planned phase sequence for Crop Diversification Rotation Study (CDRS) from 2004-2007.

Crop System	Rotation
1. ORG	(1) AWP manure - (2) W Wheat - (3) S Lentil - (4) Barley
2. PFP	(1) AWP manure - (2) W Wheat - (3) S Lentil - (4) Barley
3. NTW -L	(1) W Pea - (2) W Wheat - (3) Dormant Mustard - (4) W Triticale
4. NTW-H	(1) W Pea - (2) W Wheat - (3) Dormant Canola - (4) W Triticale
5. NTS-L	(1) S Pea - (2) S Wheat - (3) Mustard - (4) S Triticale
6. NTS-H	(1) S Pea - (2) S Wheat - (3) Canola - (4) S Triticale
7. NTD-L	(1) S Pea - (2) W Wheat - (3) Sunflower - (4) Open-pollinated corn
8. NTD-H	(1) S Pea - (2) W Wheat - (3) Sunflower - (4) Hybrid Corn
9. NTCW	(1) S Wheat- (2) W Wheat

[a] NAT=natural, L=low fertilizer rate, H=high fertilizer rate.

Table A.3. Planned phase sequence for Greenhouse Gas Rotation Study (GGRS) from 2002-2007.

Crop System[a]	Rotation
1. FW (CT)	Fallow-W Wheat
2. FW (NT)	Fallow-W Wheat
3. CW	S Wheat-W Wheat
4. SP-WW	S Pea-W Wheat
5. WP-WW	W Pea-W Wheat
6. WPF-WW	AWP (forage)-W Wheat
7. WPF-SW	AWP (forage)-S Wheat
8. ORG	AWP (manure)-S Wheat

[a] FW=fallow-wheat, CT=conventional till, NT=no-till, CW=continuous wheat, S=spring, W=winter, WPF=winter pea forage, ORG=organic, AWP=Austrian winter pea.

Table A.4. Crop Diversification Rotation Study (CDRS) mean soil total nitrogen (kg ha-1) by depth and combined depths (0-30 cm and 0-60 cm) for each crop system.

Crop System	n	Soil depth (cm)				
		0-15	15-30	30-60	0-30	0-60
ORG	8	2606	1893	1561[a]	4517	6086[a]
NAT	8	2653	1816	1581	4470	6051
NTW-L	8	2651	1895[a]	1940	4519[a]	6490[a]
NTW-H	8	2905	1835	1728	4740	6468
NTS-L	8	2606	1885	1784	4491	6275
NTS-H	8	2696	1819	1571	4515	6086
NTD-L	8	2571	1869	1997	4441	6437
NTD-H	8	2762	1756	1698	4517	6215
CW	4	2706	1806	1774	4512	6286
Source of variation	*df*	*P-values*				
Crop System	8	0.33	0.85	0.27	0.76	0.59

[a] Data point missing; n=7.

Table A.5. Crop Diversification Rotation Study (CDRS) mean soil total carbon (Mg ha^{-1}) by depth and combined depths (0-30 cm and 0-60 cm) for each crop system.

Crop System	n	Soil depth (cm)				
		0-15	15-30	30-60	0-30	0-60
ORG	8	25.90	23.57	92.61[a]	50.62	142.62[a]
NAT	8	26.80	25.58	87.56	52.38	139.94
NTW-L	8	27.14	19.59[a]	84.88	46.86[a]	129.72[a]
NTW-H	8	27.35	20.97	84.17	48.33	132.49
NTS-L	8	28.39	21.79	80.72	50.18	130.90
NTS-H	8	26.19	26.11	91.92	52.30	144.22
NTD-L	8	27.64	20.20	76.16	47.85	124.01
NTD-H	8	25.50	19.65	83.10	45.15	128.25
CW	4	27.06	18.48	77.53	45.54	123.07
Source of variation	*df*	*P-values*				
Crop System	8	0.17	0.13	0.25	0.33	0.22

[a] Data point missing; n=7.

Table A.6. Greenhouse Gas Rotation Study (GGRS) mean soil total nitrogen (kg ha^{-1}) by depth and combined depths (0-30 cm and 0-60 cm) for each crop system.

Crop System	n	Soil depth (cm)				
		0-10	10-20	20-30	30-60	0-30
1) FW (CT)	8	1523c	1510bcd	1176c	2128	4209c
2) FW (NT)	6[a]	1577abc	1448d	1162c	2133	4187c
3) CW	8	1607abc	1590abc	1261abc	2214	4458ab
4) SP-WW	8	1699a	1477cd	1215bc	2203	4390bc
5) WP-WW	8	1629ab	1642a	1334a	2363	4606a
6) WPF-WW	8	1687ab	1612ab	1262abc	2140	4561ab
7) WPF-SW	8	1588bc	1599ab	1326a	2229	4513ab
8) ORG	4	1671ab	1654a	1333a	2371	4658a
LSD		102	119	107	n.s.	212
Source of variation	*df*			*P-values*		
Crop system	7	0.02	0.01	<0.01	0.55	<0.01
N fert [crop system]	7	0.70	0.52	0.86	0.98	0.97

[a] Data point missing from block 1 plot; n=6

Table A.7. Greenhouse Gas Rotation Study (GGRS) mean soil total nitrogen (kg ha^{-1}) for combined 0-30 cm depth by fertilizer rate for each crop system. There were no significant differences among fertilizer rate treatments.

Crop System	n	Fertilizer Rate	
		Low	High
1) FW (CT)	8	4178	4240
2) FW (NT)	6	4126	4156
3) CW	8	4453	4463
4) SP-WW	8	4356	4425
5) WP-WW	8	4538	4674
6) WPF-WW	8	4611	4511
7) WPF-SW	8	4495	4531

Table A.8. Greenhouse Gas Rotation Study (GGRS) mean soil total carbon (Mg ha^{-1}) by depth and combined depth (0-30 cm) for each crop system.

Crop System	n	Soil depth (cm)				
		0-10	10-20	20-30	30-60	0-30
1) FW (CT)	8	16.23	15.88	17.51	105.69	49.62
2) FW (NT)	8	15.54	14.75	18.58	107.77	48.87
3) CW	8	15.18	14.85	13.32	105.54	43.35
4) SP-WW	8	15.21	13.43	14.36	100.37	43.00
5) WP-WW	8	16.83	15.45	16.89	98.76	49.17
6) WPF-WW	8	17.12	15.06	16.54	101.75	48.73
7) WPF-SW	8	14.81	13.78	14.70	98.73	43.28
8) ORG	4	15.75	13.82	14.47	98.72	44.03
LSD		n.s.	n.s.	n.s.	n.s.	n.s.
Source of variation	*df*			*P-values*		
Crop system	7	0.15	0.16	0.17	0.90	0.22
N fert[crop system]	7	0.99	0.97	0.99	0.99	0.99

References

Dusenbury MP, Engel RE, Miller PR, Lemke RL, Wallander R (2008) Nitrous oxide emissions from a northern Great Plains soil as influenced by nitrogen management and cropping systems. J Environ Qual 37 (2):542-550.

Grant CA, Peterson GA, Campbell CA (2002) Nutrient considerations for diversified cropping systems in the northern Great Plains. Agron J 94 (2):186-198.

Miller PR, Buschena DE, Jones CA, Holmes JA (2008) Transition from intensive tillage to no-tillage and organic diversified annual cropping systems. Agron J 100 (3):591-599.

Walley FL, Clayton GW, Miller PR, Carr PM, Lafond GP (2007) Nitrogen economy of pulse crop production in the northern Great Plains. Agron J 99 (6):1710-1718.

APPENDIX B

^{15}N NATURAL ABUNDANCE *B*-VALUE GREENHOUSE STUDY: QUANTIFYING *B*-VALUES FOR FIELD PEA AND LENTIL

Introduction

The purpose of Appendix B is to provide details on how B values were obtained for the calculation of biological nitrogen (N) fixation amounts by the ^{15}N natural abundance (NA) method in the field and greenhouse studies (Chaps. 2 and 3). A component of the ^{15}N natural abundance method is the *B* value, the shoot tissue $\delta^{15}N$ signature of selected legumes grown under N-free conditions. By knowing the *B* value, $\delta^{15}N$ signature in reference plant, and $\delta^{15}N$ signature in the legume of interest, the percentage of N uptake that was fixed can be calculated. In systems where all plant N is derived from N fixation, an effectively nodulated legume would usually have a whole plant ^{15}N value close to zero. Because it is very difficult to obtain entire root systems in field systems, however, a correction needs to be made for the within-plant fractionation of ^{14}N and ^{15}N between shoots and nodulated roots (Shearer and Kohl 1986; Unkovich et al. 2008). Numerous studies have assessed the *B* values for various legume crops; however, to our knowledge, *B* values have not been established for the specific field pea and lentil cultivars studied in this project. The objective of this greenhouse experiment was to obtain *B* values for Austrian winter pea (*Pisum sativum L.* cv. Melrose), spring pea (*P. sativum* cv. Arvika), and spring lentil (*Lens culinaris* cv. Richlea).

Materials and Methods

This greenhouse experiment was carried out in the Plant Growth Center at Montana State University-Bozeman in summer 2009. The experiment consisted of three legume crops (Austrian winter pea, spring pea, and spring lentil) and two termination

timing treatments (flower or pod; see Chap. 2 for termination timing protocol). Seeds were from the same lots used for the Amsterdam field study (Chap. 2). Each treatment was replicated five times.

Greenhouse Management and Sampling

Plants were grown in pots (20.3-cm diam. by 15.2-cm depth) filled with approximately 1.75 kg 20/30 medium grit sand (pH_{H2O}: 6.1, EC: 0.08 dS m^{-1} [1:1 sand:water]). Seeds were sown at two times the field seeding rate for each species and thinned 14 d after emergence (DAE) to match field seeding rates, resulting in three pea plants per pot and four lentils per pot. Rhizobia inoculum (same as field inoculum) was placed directly with the seeds to ensure contact. To achieve proper flowering, winter pea pots were vernalized 14 DAE in a growth chamber at 4^{o}C for 21 d. Watering regimes alternated between an N-free modified Hoagland's nutrient solution and charcoal-filtered tap water throughout the growing period (<17 µg NO_3-N L^{-1}). Pots were watered to saturation when soil water content by mass fell to 75% of the greenhouse field capacity which was determined by weighing plant-free pots 24 h after watering to full saturation. Pot arrangement was randomized at the onset of the experiment and systematically rotated on a weekly basis thereafter. Replicates of each crop type were terminated (clipped) and harvested when each pot in a treatment had a minimum of one full bloom or one full flat pod. Both shoot and root (including nodules) were harvested from each pot.

All statistical analyses were accomplished with JMP 8.0 statistical software (SAS Institute 2010). Analysis of variance (ANOVA) was performed to determine significant treatment differences between termination times at $P<0.05$.

Results and Discussion

$\delta^{15}N$ Values

Mean shoot and root $\delta^{15}N$ values for each crop by termination time are presented in Table B.1. There was no termination timing effect on shoot $\delta^{15}N$ values. Averaged across timings, $\delta^{15}N$ values (*B*-values) were -0.71 for lentil, -0.73 for pea, and -0.63 for Austrian winter pea. These values were within the range of published *B*-values for field pea and lentil cultivars (Unkovich et al. 2008).

Roots for both pea cultivars and lentil were enriched in ^{15}N ($\delta^{15}N > 0$). This is commonly found in ^{15}N studies due to less movement of ^{15}N to the aboveground biomass (Unkovich et al. 2008). There was a significant termination time effect on root $\delta^{15}N$ content. Roots were less enriched in ^{15}N at pod stage than flower for all legume species. From observation, plants were physically stressed at pod stage and ^{15}N –enriched root nodules may have begun sloughing at this stage relative to flower stage. Mean biomass and tissue N concentrations are presented in Table B.2 as supplemental data.

Table B.1. Mean shoot and root $\delta^{15}N$ content of three legume species grown under nitrogen-free conditions and terminated at flower or pod stage. Mean seed $\delta^{15}N$ content is provided as supplemental data and was not included in statistical analysis.

Legume species	Shoot $\delta^{15}N$		Root $\delta^{15}N$		Seed $\delta^{15}N$
	Flower	Pod	Flower	Pod	
Spring lentil	-0.74	-0.69	3.18	1.37	-0.31
Spring pea	-0.70	-0.76	2.81	1.68	0.37
Austrian winter pea	-0.58	-0.67	2.23	0.57	-0.29
Source of variation		*P-values*			
Termination time (T)	0.56		<0.01		

Table B.2. Mean shoot and root biomass and tissue N concentrations of three legume species grown under nitrogen-free conditions and terminated at flower or pod stage.

Legume species	Shoot biomass (g pot^{-1})		Root biomass (g pot^{-1})		Shoot N (g kg^{-1})		Root N (g kg^{-1})	
	Flower	Pod	Flower	Pod	Flower	Pod	Flower	Pod
Spring lentil	0.47	0.74	0.37	0.56	25.4	19.9	18.1	15.9
Spring pea	1.29	1.62	0.56	0.65	30.0	22.0	21.7	17.4
Austrian winter pea	1.36	1.56	0.66	0.63	26.3	23.4	23.1	20.2

References

Shearer G, Kohl DH (1986) N-2 fixation in field settings - estimations based on natural N-15 abundance. Aust J Plant Physiol 13 (6):699-756.

Unkovich M, Herridge DF, Peoples MB, Cadisch G, Boddey RM, Giller KE, Alves B, Chalk PM (2008) Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR Monograph. Australian Centre for International Agricultural Research, Canberra, AU.

APPENDIX C

ASSESSING NITROGEN FIXATION OF PEA AND LENTIL WITH A NON-NODULATING PEA REFERENCE PLANT

Introduction

The overall intent of the legume green manure tillage (LGMT) study was to examine the effects of termination method, herbicide and/or tillage, on legume green manure (LGM) nitrogen (N) contributions to a subsequent crop (Burgess, unpublished). Yet, for my purposes, the LGMT study provided an additional opportunity to further test the effect of species, methodology, reference plant selection, and spatial heterogeneity on N fixation estimates as compared with the adjacent Amsterdam study (no-till green manure, NTGM-2009) presented in Chap. 2.

Materials and Methods

Field Description and Experimental Design

This study was established directly north of the NTGM-2009 study. The site was established on barley stubble with at least 3 yr of previous no-till management. Soil type was Amsterdam silt loam (frigid Typic Halpustoll) and soil characteristics are given in Table C.1. Climate data was reported in Chapter 2.

The study was a randomized complete block design (RCBD) consisting of four replicate blocks of 20 plots each; however, due to treatments not needed for this portion of the project, only a subset of either six or seven plots per block were used for data collection (Fig. C.1). Each plot was 8 x 8 m. The main plot factor was legume crop: spring pea (*Pisum sativum* cv. Arvika), spring lentil (*Lens culinaris* cv. Richlea), or a non-nodulating spring pea (*Pisum sativum* cv. Sparkle) selected to serve as the reference plant for N fixation measurements. Two non-nodulating pea (NNP) treatments, one

receiving additional N fertilizer (NNP+) and one receiving no additional fertilizer (NNP-), were established to provide a comparison of reference plants under two growth-affecting (N adequate and N deficient) conditions for measurement of N fixation. A main assumption of the N fixation method is that reference plant and legume plant roots will explore similar soil volumes. It was observed in previous research that NNP grew poorly without additional N, hence the fertilized NNP treatment was added to this study. Fallow treatments were included as controls. Sampled plots were selected based on proximity of each N-fixing legume plot to both a NNP+ and NNP- plot. Termination method (spray or till) was not part of the selection process and was not considered a treatment effect as all data were collected prior to the tillage treatment.

Crop Management, Field and Laboratory Measurements, and N Fixation Calculations

All plots were planted 13 Apr. 2009. Seeding rates, row width, and fertilization rates were identical to NTGM, with the exception that NNP+ plots received an additional 60 kg N ha^{-1}. Herbicide terminated plots were sprayed on 28 June 2009 with glyphosate plus AMS as described for the NTGM study. Biomass from each legume plot was harvested 3 d later on 1 July. Biomass sampling was identical to NTGM 2009 for all plots (1-m^2 sample). Soil was sampled at seeding (2 Apr.) and on the day following harvest from two locations near the centerline of each plot approximately 2.25 m in from each north and south plot boundary. The two cores were composited by depth. Soil sampling depths and collection and laboratory measurements of soil and plant material were identical to those described for NTGM 2009 (Chap. 2).

Similar to the NTGM study, the N difference (ND) and ^{15}N natural abundance (NA) methods were employed to quantify N fixation with NNP+ and NNP- serving as reference plants for each method. Equations and calculations for the NA method were presented in Chapter 2 and the same *B*-values used in this study. The ND equation was modified from the one presented in Chap. 2 to account for soil N at seeding and fertilizer application in the NNP+ plots (Eq. 1).

$$\text{N fixed} = \left(\text{Plant N}_{\text{leg}} - \text{Plant N}_{\text{ref}}\right) - \left(\Delta\text{Soil N}_{\text{leg}} - \Delta\text{Soil N}_{\text{ref}}\right) + \left(\text{Fertilizer N}_{\text{ref}}\right) \quad [1]$$

where ΔSoil N = soil N (harvest) – soil N (seeding) and

Fertilizer N_{ref} = available N fertilizer applied to NNP+ plots above that applied to N-fixing legume plots which was assumed to be approximately 2/3 of the amount of N fertilizer applied (40 kg N ha^{-1}).

Statistical Analysis

All statistical analyses were performed using JMP 8.0 statistical software (SAS Institute, 2010). Simple linear regression was used to compare amounts of N fixed between N fixation methods. Analysis of variance (ANOVA) was used to compare N fixation estimates by method and $\delta^{15}N$ values between the LGMT and NTGM studies. Block was considered a random effect and legume species and study were fixed effects.

Results and Discussion

Comparison of N Fixation By Method and Reference plant

Mean aboveground biomass, shoot N uptake, and $\delta^{15}N$ values for each legume crop are provided in Table C.2. Estimates of N fixed by pea and lentil for each N fixation

method using NNP+ or NNP- as a reference plant are given in Table C.3. There was better agreement in N fixation estimates between the ND and NA methods when NNP- was used as the reference plant than NNP+ (Fig. C.2). There was higher variability in N fixation values for both methods when NNP+ was used as the reference plant and three of 16 NNP+ measurements resulted in negative N fixation. Averaged across plots, NNP+ had lower $\delta^{15}N$ values than NNP- (P=0.001), suggesting either the applied N fertilizer was depleted in ^{15}N or the NNP+ plants were accessing a soil N pool not accessed by the NNP- plots. Because application of N fertilizer can cause differences in N mineralization, plant N uptake, and the $\delta^{15}N$ of soil available N to occur, it is not recommended to apply N fertilizer to the reference plant when using either of these methods (Unkovich et al. 2008). Thus, the following section focuses only on N fixation estimates using NNP- as the reference plant.

Comparison of N Fixation Between Adjacent Studies

The counterpart treatment to the LGMT study in the 2009 NTGM study was spring-planted pea and lentil terminated at flower. Spring wheat was used as the primary reference plant in the NTGM study for both the ND and NA methods. Incidental weeds (*Bromus tectorum*) were also collected from NTGM plots to provide an additional reference plant for the NA method. For the ND method, wheat as a reference plant (NTGM) resulted in higher estimates of N fixed than NNP- as a reference plant (Table C.4.). Specifically, NTGM estimates of N fixed were 33 and 27 kg N ha^{-1} higher for pea and lentil, respectively, than LGMT estimates. Without the addition of N fertilizer, the NNP- plants were not very robust and more NO_3 may have been immobilized, denitrified,

or leached below the 0.9-m soil sampling depth in the NNP- plots than the NTGM wheat plots. A loss of unaccounted soil NO_3 at harvest in the reference plots would lower the amount of N fixed calculated by the ND method; this may have contributed to lower N fixation estimates by the ND method in the LGMT study.

There was better agreement among N fixation estimates by the NA method between the two studies. Mean $\delta^{15}N$ values for NNP- (1.63‰) were similar to wheat (1.51‰) and weeds (1.58‰) at flower termination. Mean lentil $\delta^{15}N$ values were also similar between the two studies (0.51‰ vs. 0.61‰). Mean pea $\delta^{15}N$ values, however, were significantly lower in the LGMT study (0.13‰) than NTGM study (0.49‰) (P=0.002). It is unclear why there was a difference in pea $\delta^{15}N$ values between studies and not reference plant or lentil $\delta^{15}N$ values.

Table C.1. General soil properties for 2009 legume green manure tillage study measured at spring seeding. Soil characteristics are for 0-0.15 m depth, unless otherwise stated.

Soil property	2009
Soil texture	Clay loam/loam
Soil pH	6.6-8.1
Organic carbon (g kg^{-1})[a]	14
NO_3-N (kg N ha^{-1})[ab]	26
Olsen P (mg kg^{-1}) [a]	29
K (mg kg^{-1}) [ac]	440
SO_4-S (mg kg^{-1}) [a]	8

[a] Analysis averaged across 12 composited samples of four subsamples per site.
[b] Measured to 0.9-m depth.
[c] Ammonium acetate extractable K.

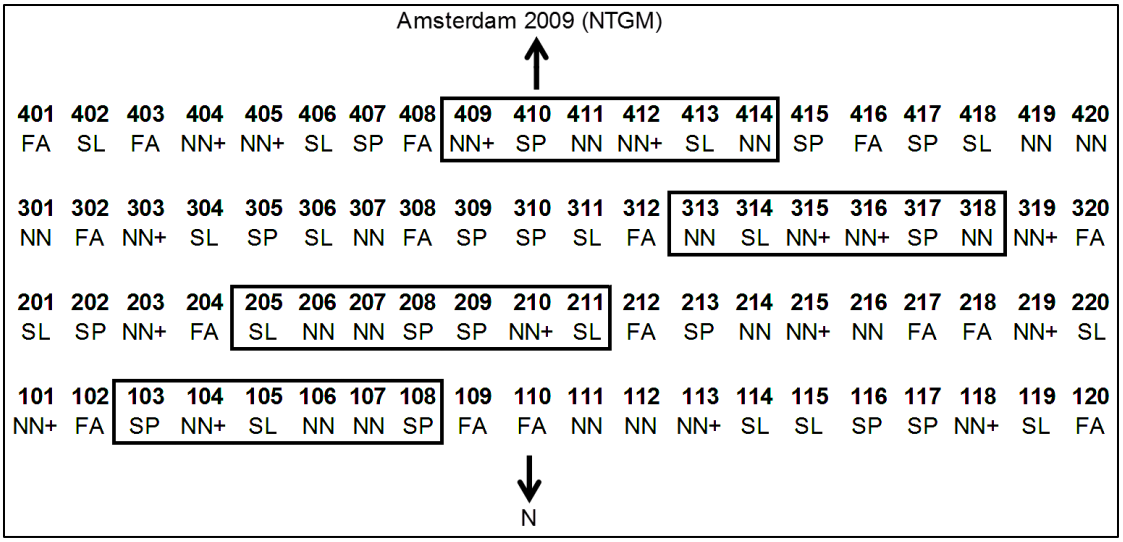

Figure C.1. Legume green manure tillage (LGMT)-2009 study plot map. Outlined areas denote plots sampled for measurement of nitrogen fixation. The Amsterdam 2009/no-till green manure (NTGM) study was located directly south of LGMT.

Table C.2. Mean aboveground dry biomass, shoot N uptake, and $\delta^{15}N$ value by legume species for select plots.

Legume crop	Biomass (Mg ha^{-1})	Shoot N (kg N ha^{-1})	$\delta^{15}N$ (‰)
Pea	3.13	104.6	0.13
Lentil	1.15	40.1	0.60
NNP+	2.39	60.5	0.93
NNP-	1.31	19.8	1.63

Table C.3. Mean nitrogen fixed (kg N ha^{-1}) for pea and lentil as calculated by the N difference (ND) and ^{15}N natural abundance (NA) methods using non-nodulated pea unfertilized (NNP-) or fertilized (NNP+) as the reference plant for both methods.

	N fixed (kg N ha^{-1})			
	ND		NA	
Legume crop	NNP-	NNP+	NNP-	NNP+
Pea	54.1	74.1	59.7	35.0[a]
Lentil	9.3	11.6	17.3	14.7

[a] Extreme negative outlier removed, n=3.

Table C.4. Comparison of mean nitrogen (N) fixed by spring-planted pea and lentil terminated at flower as calculated by the N difference (ND) and ^{15}N natural abundance (NA) methods for the legume green manure tillage (LGMT) and no-till green manure (NTGM) studies.

	N fixed (kg N ha^{-1})				
	ND method		NA method		
Legume	LGMT[a]	NTGM[b]	LGMT[a]	NTGM-wheat[b]	NTGM-weed[c]
Pea	54.1	87.4	59.7	49.3	56.0
Lentil	9.3	36.5	17.3	22.5	22.7

[a] Unfertilized non-nodulated pea (NNP-) as reference plant.
[b] Spring wheat (*Triticum aestivum* cv. Choteau) as reference plant.
[c] *Bromus tectorum* as weed reference plant.

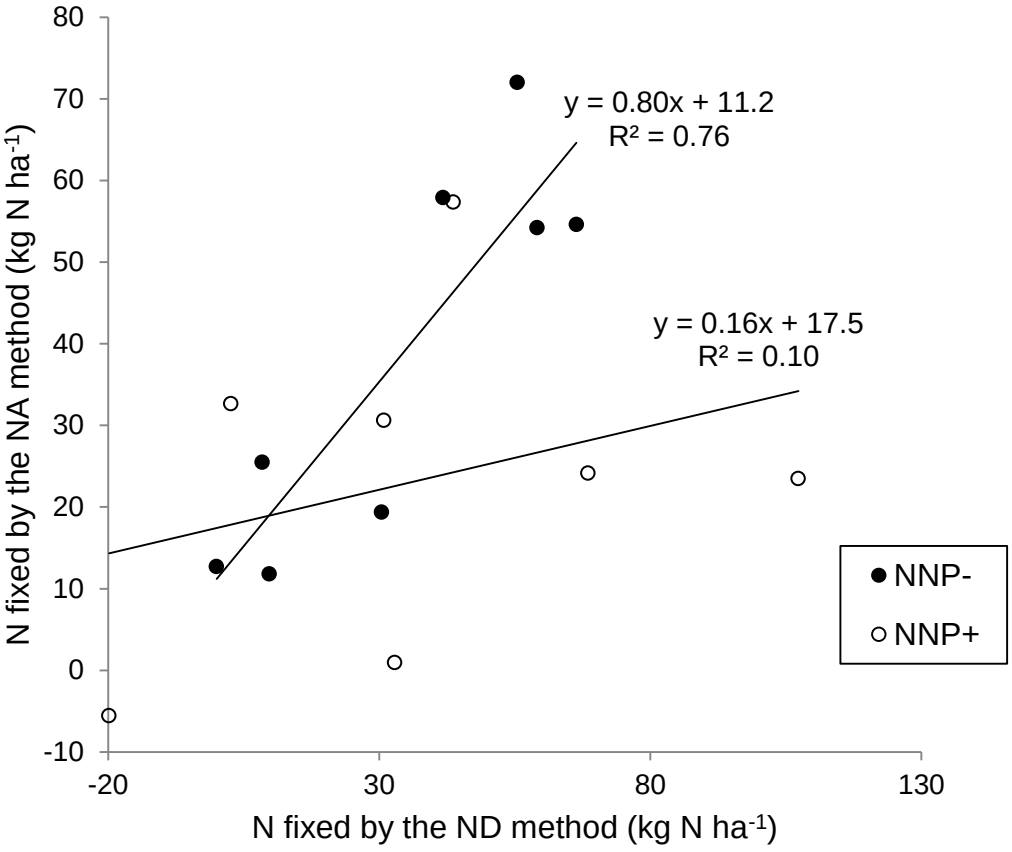

Figure C.2. Correlation between nitrogen (N) fixed determined by the N difference (ND) method and the ^{15}N natural abundance (NA) method using methods using non-nodulated pea unfertilized (NNP-, filled circles) or fertilized (NNP+, filled circles) as the reference plant for both methods.

References

Unkovich M, Herridge DF, Peoples MB, Cadisch G, Boddey RM, Giller KE, Alves B, Chalk PM (2008) Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR Monograph. Australian Centre for International Agricultural Research, Canberra, AU.

APPENDIX D

AMSTERDAM ADDITIONAL TABLES AND FIGURES

101	**Spring**	**Lentil**	**Pod**
102	**Spring**	**Pea**	**Flower**
103	**Spring**	**Pea**	**Pod**
104	**Late Summer**	**Lentil**	**Natural**
105	**Spring**	**Lentil**	**Intermediate**
106	**Summer**	**Lentil**	**Flower**
107	**Fallow**		
108	**Summer**	**Pea**	**Flower**
109	**Summer**	**Lentil**	**Natural**
110	**Spring**	**Pea**	**Intermediate**
111	**Summer**	**Pea**	**Natural**
112	**Spring**	**Lentil**	**Flower**
113	**Late Summer**	**Pea**	**Natural**

201	**Spring**	**Lentil**	**Intermediate**
202	**Summer**	**Lentil**	**Flower**
203	**Summer**	**Lentil**	**Pod**
204	**Fallow**		
205	**Spring**	**Pea**	**Flower**
206	**Spring**	**Pea**	**Pod**
207	**Spring**	**Pea**	**Intermediate**
208	**Late Summer**	**Pea**	**Natural**
209	**Summer**	**Pea**	**Flower**
210	**Summer**	**Pea**	**Pod**
211	**Spring**	**Lentil**	**Flower**
212	**Late Summer**	**Lentil**	**Natural**
213	**Spring**	**Lentil**	**Pod**

301	**Summer**	**Pea**	**Flower**
302	**Spring**	**Lentil**	**Intermediate**
303	**Spring**	**Lentil**	**Flower**
304	**Late Summer**	**Lentil**	**Natural**
305	**Spring**	**Pea**	**Intermediate**
306	**Summer**	**Lentil**	**Flower**
307	**Summer**	**Pea**	**Pod**
308	**Summer**	**Lentil**	**Pod**
309	**Spring**	**Pea**	**Pod**
310	**Spring**	**Lentil**	**Pod**
311	**Late Summer**	**Pea**	**Natural**
312	**Spring**	**Pea**	**Flower**
313	**Fallow**		

401	**Summer**	**Pea**	**Pod**
402	**Late Summer**	**Pea**	**Natural**
403	**Spring**	**Pea**	**Flower**
404	**Summer**	**Pea**	**Flower**
405	**Summer**	**Lentil**	**Pod**
406	**Spring**	**Lentil**	**Flower**
407	**Summer**	**Lentil**	**Flower**
408	**Late Summer**	**Lentil**	**Natural**
409	**Spring**	**Lentil**	**Pod**
410	**Spring**	**Pea**	**Pod**
411	**Fallow**		
412	**Spring**	**Lentil**	**Intermediate**
413	**Spring**	**Pea**	**Intermediate**

Figure D.1. 2009 plot map for no-till green manure study at Amsterdam.

101	**Fallow**		
102	**Spring**	**Pea**	**Pod**
103	**Winter**	**Lentil**	**Pod**
104	**Spring**	**Pea**	**Flower**
105	**Summer**	**Lentil**	**Flower**
106	**Spring**	**Lentil**	**Pod**
107	**Summer**	**Lentil**	**Pod**
108	**Winter**	**Pea**	**Pod**
109	**Summer**	**Pea**	**Pod**
110	**Spring**	**Lentil**	**Flower**
111	**Summer**	**Pea**	**Flower**
112	**Winter**	**Lentil**	**Flower**
113	**Winter**	**Pea**	**Flower**

201	**Summer**	**Pea**	**Pod**
202	**Fallow**		
203	**Winter**	**Pea**	**Flower**
204	**Summer**	**Lentil**	**Pod**
205	**Winter**	**Lentil**	**Pod**
206	**Spring**	**Lentil**	**Flower**
207	**Summer**	**Lentil**	**Flower**
208	**Spring**	**Lentil**	**Pod**
209	**Spring**	**Pea**	**Flower**
210	**Summer**	**Pea**	**Flower**
211	**Spring**	**Pea**	**Pod**
212	**Winter**	**Pea**	**Pod**
213	**Winter**	**Lentil**	**Flower**

301	**Summer**	**Lentil**	**Flower**
302	**Spring**	**Lentil**	**Pod**
303	**Winter**	**Pea**	**Pod**
304	**Spring**	**Lentil**	**Flower**
305	**Winter**	**Lentil**	**Pod**
306	**Winter**	**Pea**	**Flower**
307	**Spring**	**Pea**	**Pod**
308	**Summer**	**Pea**	**Pod**
309	**Summer**	**Lentil**	**Pod**
310	**Summer**	**Pea**	**Flower**
311	**Winter**	**Lentil**	**Flower**
312	**Fallow**		
313	**Spring**	**Pea**	**Flower**

401	**Spring**	**Lentil**	**Flower**
402	**Winter**	**Lentil**	**Pod**
403	**Summer**	**Lentil**	**Flower**
404	**Winter**	**Lentil**	**Flower**
405	**Spring**	**Pea**	**Pod**
406	**Winter**	**Pea**	**Flower**
407	**Fallow**		
408	**Winter**	**Pea**	**Pod**
409	**Summer**	**Lentil**	**Pod**
410	**Summer**	**Pea**	**Flower**
411	**Summer**	**Pea**	**Pod**
412	**Spring**	**Pea**	**Flower**
413	**Spring**	**Lentil**	**Pod**

Figure D.2. 2010 plot map for no-till green manure study at Amsterdam.

Table D.1. Mean soil NO_3-N (0-0.9 m depth) at harvest for lentil, pea, wheat, and fallow plots at each planting/termination time for 2009 and 2010 Amsterdam sites.

Planting[a]	Termination	Soil NO_3-N (kg N ha^{-1})			
		Pea	Lentil	Wheat	Fallow
		----------2009----------			
Spring[a]	Flower	11.3	14.3	10.9	28.3
	Int	13.8	21.0	9.1	29.0
	Pod	12.2	11.0	9.2	27.9
		----------2010----------			
Spring	Flower	30.4	23.6	18.8	na[b]
	Pod	30.7	32.7	15.1	na
Summer	Flower	23.6	23.1	19.3	na
	Pod	14.6	11.4	12.6	na

[a] Only spring-planting treatments were sampled in 2009.
[b] Fallow not sampled or not analyzed for soil NO_3-N.

Table D.2. Mean soil water (0-0.9 m depth) at harvest for lentil, pea, wheat, and fallow plots at each planting/termination time for 2009 and 2010 Amsterdam sites.

Planting[a]	Termination	Soil water (cm)			
		Pea	Lentil	Wheat	Fallow
		----------2009----------			
Spring[a]	Flower	14.1	16.2	16.7	21.1
	Int	12.2	16.4	15.2	22.0
	Pod	11.5	12.4	14.9	21.2
		----------2010----------			
Spring	Flower	17.7	20.0	20.8	na[b]
	Pod	13.4	15.0	19.4	23.0
Summer	Flower	13.0	14.7	14.0	21.3
	Pod	12.0	12.4	12.8	na[b]

[a] Only spring-planting treatments were sampled in 2009.
[b] Fallow not sampled.

Table D.3. $\delta^{15}N$ content of soil NO_3-N and plant tissue from select Amsterdam 2009 plot/treatments (Trmt). Selected plots were chosen to represent soil and plant tissue $\delta^{15}N$ content at flower (Flw), intermediate (Int), and pod terminations. Analyzed soil samples are from spring-planted plots at seeding (legume and wheat plots) and spring-planted plots at pod termination (fallow plots). No plants were harvested from fallow plots.

Plot #-Trmt	$\delta^{15}N$ of soil NO_3-N[a] (‰)			Plant $\delta^{15}N$	Weed $\delta^{15}N$ [b]
	0-30 cm	30-60 cm	60-90 cm	(‰)	(‰)
102-Pea-Flw[c]	2.14	7.02	5.85	0.7	2.29
102-Wheat-Flw	0.59	3.70	-2.16	2.25	--
110-Pea-Int[c]	5.53	1.71	-4.89	0.72	4.62
110-Wheat-Int	1.82	0.34	13.58	0.69	1.55
312-Pea-Flw[d]	-1.68	-0.60	2.45	0.33	1.84
410-Pea-Pod[d]	0.76	-0.99	0.06	0.86	--
107-Fallow	0.54	3.67	4.74		
204-Fallow	2.02	3.10	1.15		
313-Fallow	2.13	0.09	1.87		
411-Fallow	3.11	1.46	-0.81		

[a] Soil NO_3-N extraction method: 5 g dried soil 50 ml^{-1} of 0.05 M KCl, shaken for 30 min, filtered (Whatman #42), and frozen. ^{15}N analysis of extractable NO_3-N was done by bacteria denitrification assay.

[b] *Bromus tectorum*, when present in legume plot or wheat strip.

[c] Soil sample from south-side of plot (next to wheat strip).

[d] Soil sample from north-side of plot.

APPENDIX E

GREENHOUSE STUDY: STERILIZED SOIL EXPERIMENT

Introduction

The purpose of Appendix E is to present findings from a sterilized soil greenhouse experiment that paralleled the greenhouse study discussed in Chapter 3. The initial design for the greenhouse experiment included a soil treatment (unsterilized and sterilized) to assess the effect of phosphorus (P) fertilization and arbuscular mycorrhizae fungus (AMF) inoculation on pea and lentil growth and N fixation in the presence and absence of a native AMF community; however, the sterilization process altered the chemistry of the sterilized soil compared to the unsterilized soil. Subsequently, the effect of soil treatment on legume growth and N fixation was potentially confounded by differences in soil chemistry between the two soil treatments. To avoid introducing a confounding variable to the experiment, soil sterilization was excluded as an experimental variable and results from the two soil sterilization treatments were analyzed independently.

Materials and Methods

Soil sterilization

The unsterilized and sterilized experiments were carried out simultaneously in the same greenhouse room and with the exception of some set-up differences related to the soil sterilization process, the experimental design, treatment application, seeding rate, study maintenance, and harvest schedule were identical for the two experiments (see Chapter 3). Thus, only set-up differences are reported here. The soil:sand mixture was sterilized by aerated steam pasteurization (twice at 70^{o}C for 60 min with a 24 h resting period). Post-sterilization, the covered soil was allowed to cool and equilibrate for 4 d.

Because of the steam sterilization process, the water content of the sterilized soil was near field capacity (approximately 18 g water per 100 g soil). To more closely match the unsterilized soil, sterilized soil was thoroughly mixed, moved to sterilized (10% bleach solution) soil bins, covered with Reemay® fabric (to minimize air-borne AMF contamination, but allow for evaporation), and allowed to air-dry for an additional 4 d prior to potting. At 4 d after seeding, 25 mL of microbial wash, prepared by filtering a 1:5 [w,v] unsterilized soil:distilled water slurry through an 11-µm filter (modified from (1993) was added to each sterilized soil pot to provide a non-AMF background soil microbial population.

Soil N Mineralization Study

Following soil sterilization, a small time-series study was conducted to compare N between the unsterilized soil and sterilized soil with a microbial wash. Approximately 4 d following sterilization, approximately 1 kg of sterilized soil was collected from four different areas in the sterilizing cart. Four 5 g subsamples of soil were removed from each soil treatment for baseline (Day 0) soil NO_3-N content. For each soil type, two 15-cm greenhouse pots per were filled with 1.5 kg of well-mixed soil. After potting, sterilized soil pots received 100 mL of water plus 25 mL microbial wash and unsterilized pots received 150 mL of water. The difference in water applications between soil treatments was to compensate for higher soil moisture content in the sterilized soil due to the steam sterilization process. Pots were covered with plastic wrap secured by a rubber band (to maintain soil moisture) and placed on a greenhouse bench. Soil was sampled from each pot on days 6, 11, and 19 after potting by removing the soil from the pot, mixing the soil

thoroughly with a clean spatula, subsampling 5 g of soil, returning the mixed soil to its original pot, re-covering the pot with plastic wrap, and returning the pot to the greenhouse bench. Subsampled soil was dried (50^{o}C, 72 h) immediately following sampling and stored until the end of the experiment. At the end of the experiment, soil from each sampling date was extracted for soil available NO_3-N (plus NH_4-N for Day 19 samples) with 1 M KCl as outlined by Bundy and Meisinger (1994). Extracts were analyzed using a flow injection analyzer (Lachat Instruments Inc., Loveland).

Statistical Analysis

All statistics on the main study were performed using JMP 8.0 statistical software (SAS Institute, 2010). Analysis of variance (ANOVA) was performed to examine significant treatment differences and interactions (P<0.05). Plant species, P fertilizer, and AMF inoculation were considered fixed effects.

Results and Discussion

Soil N Mineralization Study

The effects of soil sterilization on soil available NO_3-N (plus NH_4-N for Day 19 samples only) are shown in Table E.1. Soil NO_3-N levels steadily increased in the unsterilized soil through Day 19, indicating increased soil N mineralization over the course of the experiment. In contrast, soil NO_3-N levels in the sterilized soil decreased with subsequent sampling dates from Day 0. By Day 19, soil NO_3-N in the sterilized pots was only 26% of soil NO_3-N levels in the unsterilized soil; however, NH_4-N levels at the end of the experiment were substantially higher in the sterilized soil than the unsterilized

soil. By Day 19, total available N (NO_3 + NH_4) was 9 mg N kg-soil^{-1} higher in the sterilized soil compared to the unsterilized soil. In addition, there was a high amount of variability in individual Day 0 soil NO_3-N levels from the sterilized treatment (sampled from four different locations in soil cart), suggesting sterilization effects on soil N were not uniform.

Soil sterilization processes can have severe effects on soil N dynamics that can affect subsequent plant growth, and likely N fixation. The most notable change to soil N is an increase in soil ammonium (NH_4-N) which is most often attributed to decomposition of microbial debris (Gelsomino et al. 2010; Yamamoto et al. 2008). For steam-sterilized soils, however, a physical release of NH_4-N from soil organic fractions and microbial debris can occur through deamination processes (removal of an amine group from a molecule) during soil heating (Yamamoto et al. 2008). This can result in a large spike in NH_4-N levels immediately following sterilization that can remain high for a number of weeks depending on plant uptake of available NH_4-N or re-colonization of nitrifying bacteria in the soil. Soil NO_3-N levels remained low in the sterilized soil throughout the 19 d period despite adding a microbial wash that presumably included nitrifying bacteria. The slight increase in NO_3-N levels by Day 19 compared to previous sampling dates may indicate a slow increase in nitrification. The high NH_4-N:NO_3-N ratio in the sterilized treatments at the end of this experiment is indicative of anoxic soil conditions; the soil was moist and would have contained high concentrations of readily decomposable organic material from lysed microbial cells, possibly sufficient to depress oxygen levels. Only soil available N was measured in the post-sterilized soils, so it is

unknown what other soil chemistry changes may have occurred due to the steam sterilization process. In addition to NH_4-N, large increases in exchangeable potassium (K^+) and manganese (Mn^{2+}) have been observed following steam sterilization (Gelsomino et al. 2010).

Biomass and Nutrient Uptake

There was a positive P fertilization effect on shoot biomass and N and P uptake for lentil and pea (Table E.2). Inoculation had a significant positive effect on lentil N uptake in the fertilized treatments. There were no inoculation effects or interactions on any of the other measured parameters for either species. Averaged across inoculation treatments, lentil biomass increased 2-fold between 0P and 4P and 3-fold between 0P and 8P. For pea, biomass averaged across inoculation treatments increased 39% between 0P and 4P and 47% between 0P and 8P. *A priori* contrasts found a significant difference between inoculation treatments in the pea 4P treatments with higher biomass, N uptake, and P uptake in the AMF- than the AMF+ treatment (P=0.004). This is in contrast to findings in the unsterilized experiment where pea biomass and N uptake at the 4P rate were increased by AMF inoculation. There were no significant differences in measured parameters in the 0P and 8P pea treatments between inoculation treatments. Root colonization rates were not quantified for this experiment; however a subsample of root lengths suggests AMF colonization was poor in the inoculated treatments. Mycorrhizal features were not observed in the roots of any non-inoculated pea treatments, but a more thorough investigation would be required to rule out AMF contamination.

N Fixation

Measurement of N fixation by the ^{15}N natural abundance method was not feasible in this study as a result of greater ^{15}N enrichment in the N-fixing legumes than the non-N-fixing reference plant (non-nodulated pea). The reason why this occurred is unknown, yet appears to be related to the soil sterilization process. Averaged across fertilizer and inoculation treatments, mean pea and lentil $\delta^{15}N$ values were more than 2‰ higher in the sterilized soil experiment than mean pea and lentil $\delta^{15}N$ values in the unsterilized soil experiment. The average ^{15}N signature of available soil NO_3-N pre-sterilization was 5.60‰ (n=2). Release of NH_4-N from soil organic fractions highly-enriched in ^{15}N may have further increased the ^{15}N signature of soil available N.

A qualitative estimate of N fixation by comparing $\delta^{15}N$ values among treatments was inconclusive. In contrast to the unsterilized experiment, lentil $\delta^{15}N$ values increased with increasing P fertilization rates. This may have been due to a dilution effect of seed ^{15}N with soil ^{15}N .The 0P lentil plants were particularly small and approximately 20% of tissue N could have come from the seed which had an average $\delta^{15}N$ content of -0.31‰ (Table B.1). As discussed above, steam sterilization increased soil available N in the form of NH_4-N and high levels of available N can inhibit or delay nodulation (van Kessel and Hartley 2000). Small nodules were observed on lentil roots, but N fixation was likely minimal and a large proportion of lentil tissue N in the 4P and 8P treatments could have come from the soil. A decrease in $\delta^{15}N$ values was observed with increasing P fertilization rates for pea, but $\delta^{15}N$ levels remained high enough to question the source of plant N.

Table E.1. Soil nitrate (NO_3-N) and ammonium (NH_4-N) content (mg N kg-soil^{-1}) in unsterilized and sterilized soil sampled over the course of a 19 d incubation period. The first sampling occurred 4 d after sterilization.

	Unsterilized Soil		Sterilized Soil	
D after sterilization	NO_3-N	NH_4-N[a]	NO_3-N	NH_4-N[a]
4	7.34	--	9.68[b]	--
10	14.30	--	4.79	--
15	19.38	--	4.37	--
23	22.51	4.12	6.05	29.64

[a] Only measured for Day 19 samples.
[b] Individual soil tests from four locations in soil cart were: 16.55, 10.14, 9.32, and 2.50 mg N kg-soil^{-1}.

Table E.2. Biomass, shoot nitrogen (N) and phosphorus (P) uptake, and tissue N and P concentrations for lentil and pea under three P fertilization rates (mg P kg-soil^{-1}) without (-) or with (+) arbuscular mycorrhizae fungi (AMF) inoculum.

P Fertilizer	AMF	Biomass (g pot^{-1})	N uptake (mg pot^{-1})	P uptake (mg pot^{-1})	Tissue N (g kg^{-1})	Tissue P (g kg^{-1})	δ^{15}N (‰)
Lentil							
0	-	0.50	22.40	0.68	45.8	1.4	2.07
	+	0.42	20.40	0.80	49.4	1.5	1.53
4	-	1.00	31.92	1.66	31.9	1.6	3.03
	+	1.05	33.60	1.89	32.4	1.8	3.43
8	-	1.46	42.50	3.17	29.7	2.2	4.92
	+	1.74	51.16	3.52	30.6	2.0	4.82
Source of variation	*df*			*P-values*			
P fertilizer (P)	2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Inoculation (I)	1	0.20	0.04	0.24	0.75	0.73	0.75
P x I	2	0.37	0.20	0.71	0.95	0.13	0.86
Pea							
0	-	1.47	47.80	1.76	32.7	1.2	4.45
	+	1.42	48.05	1.68	36.5	1.2	4.74
4	-	2.72	70.93	4.47	26.0	1.6	3.72
	+	2.05	56.04	3.47	27.4	1.7	4.36
8	-	2.61	70.96	5.36	27.2	2.1	3.79
	+	2.86	74.15	6.02	25.9	2.1	3.68
Source of variation	*df*			*P-values*			
P fertilizer (P)	2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Inoculation (I)	1	0.29	0.27	0.55	0.47	0.37	0.18
P x I	2	0.4	0.73	0.21	0.26	0.85	0.41

References

Bundy LGM, J.J. (1994) Nitrogen availability indices., vol SSSA Book Ser. 5. Methods of Soil Analysis. Part 2. Biological Methods. Soil Science Society of America, Madison, WI.

Gelsomino A, Petrovicova B, Zaffina F, Peruzzi A (2010) Chemical and microbial properties in a greenhouse loamy soil after steam disinfestation alone or combined with CaO addition. Soil Biol Biochem 42 (7):1091-1100.

Johnson NC (1993) Can fertilization of soil select less mutualistic mycorrhizae? Ecol Appl 3 (4):749-757.

van Kessel C, Hartley C (2000) Agricultural management of grain legumes: has it led to an increase in nitrogen fixation? Field Crops Res 65 (2-3):165-181.

Yamamoto T, Ultra VU, Tanaka S, Sakurai K, Iwasaki K (2008) Effects of methyl bromide fumigation, chloropicrin fumigation and steam sterilization on soil nitrogen dynamics and microbial properties in a pot culture experiment. Soil Sci Plant Nutr 54 (6):886-894.

APPENDIX F

SOIL NITRATE-N VARIABILITY STUDY

Introduction

Soil testing is an important part of soil fertility management and monitoring in agricultural systems; however, spatial heterogeneity in soil nutrient content, particularly for mobile nutrients such as nitrate (NO_3), can result in a high degree of variability among soil tests across a given area. Thus, it is important to choose an appropriate number of soil samples to collect per plot that meets the precision and accuracy needs of the study while balancing the practical aspects of field sampling and sample processing. The purpose of Appendix F is to present findings from a preliminary field study designed to assess the degree of variability in soil NO_3-N levels among different sampling size protocols at the plot-scale.

Materials and Methods

Experimental Design, Soil Sampling, and Laboratory Analysis

This study was conducted at the 2010 Amsterdam (no-till green manure) study site (Chap. 2). The main Amsterdam experiment consisted of four replicate blocks with a 9.1-m alley way between blocks (Table D.2.). Three 8 by 8-m plots (one per alley way) were set up to represent the south, middle, and north ends of the field study (Fig. F.1). Within each plot, 12 sampling locations were measured and flagged in a systematic grid: locations were 2 m apart from one another south-north and 1.6 m apart from one another east-west (Fig.F.2). Soil cores (30.1-mm diam.) were extracted from each sampling location to a depth of 0.9 m and split into 0.3-m depth increments. Individual soil cores by depth were stored in plastic-coated paper bags and placed in coolers for transport from

the field to laboratory. In the laboratory, soils were weighed, dried (50^{o}C, 72 h), and reweighed to determine bulk density from gravimetric water content. Dried soil was ground (<2 mm) and subsamples were extracted for soil NO_3-N with 1 M KCl as outlined by Bundy and Meisinger (1994). Extracts were analyzed using a Lachat Flow Injection Analyzer (Lachat Instruments Inc., Loveland). Soil bulk density values were averaged by depth across all plots and used to calculate soil NO_3-N content (kg N ha^{-1}) from soil NO_3-N concentrations.

Data Analysis

Soil sampling methods for the main field experiments presented in thesis project consisted of four soil cores per plot (Amsterdam, Chap. 2; LGMT, App. C). Sampling by other researchers at the same study sites composited two soil cores per plot. For the four core method, soil cores were taken from each of the four quadrants, approximately 1.5 m and 2.5 m inside the plot from the north/south and east/west plot boundaries, respectively (Fig. D.3). For the two core method, soil sampling locations were located along the width (east-west) midline of each plot and approximately 2 m in from the north/south plot edges. To mimic these sampling methods plus a 6-core method, soil NO_3-N contents (0.9 m depth) were averaged across the following soil core groups (cores averaged per plot): 2-cores (locations 2 and 11), 4-cores (locations 1, 3, 10, and 12) or 6-cores (locations 4, 5, 6, 7, 8, and 9). A plot average was calculated by averaging all 12 cores. The absolute difference between each core group and the 12-core plot average was calculated for each plot and averaged across plots for a site average.

To increase the sample size of core group combinations, an additional five sampling location combinations (systematically assigned) for each core group (1, 2, 4, and 6 samples) in a plot were averaged and absolute differences between core averages and the 12-core average within a plot were calculated. Analysis of variance (ANOVA) was performed to determine significant differences in absolute difference from the plot average among core groups ($P<0.05$).

Results

Soil NO_3-N content (0.9-m depth) at each sampling location for each plot is presented in Table F.1. The range of measured soil NO_3-N levels among sampling locations within a plot were similar among the three plots with a low-high range difference of 35.1, 37.3, and 37.2 kg N ha^{-1} for plots 1, 2, and 3, respectively. The absolute difference between core groups (2, 4, or 6) and the plot average differed across plots (Table F.2, Fig. F.3). The 4-core average had the highest absolute difference value in plot 1, whereas it had the lowest absolute difference value in plot 3. Plot 2 had the lowest variability among core groups as all groups were within 1.4 kg N ha^{-1} of the plot average. Averaged across plots, absolute differences between each core group were similar ($P=0.72$) and differed by less than 1.5 kg N ha^{-1}.

A systematic sampling of six location combinations per core group resulted in a decrease in absolute differences from the plot average with an increase in core number (Fig. F.4). The 1-core samples resulted in the largest range of absolute differences with a low-high range of 21.7 kg N ha^{-1}. This was almost twice as high as the second highest

absolute difference range of 11.7 kg N ha^{-1} among the 2-core samples. There was a significant effect of core number on absolute difference from the plot average (Table F.3). One-core samples had the highest average absolute difference (9.7 kg N ha^{-1}). Absolute differences among the 2, 4, or 6-core groups were not significantly different from one another. It should be noted that sampling combinations were limited at six and were not randomly assigned. Spatial auto-correlation, variance associated with the 12-core plot average, and a lack of independence among core groups and the 12-core plot average were not addressed in this study, but are important issues that should be considered when reviewing this analysis.

Conclusions

Findings from this preliminary field study indicate that spatial variability at both the plot- and field-scale can affect how well soil sampling methods represent soil NO_3-N content for a given area. Increasing the number of samples per plot from 1-core to 2 or more cores decreased the absolute difference from the plot average by 48% or more. There was no difference in absolute differences among the 2, 4, and 6 core groups. In the context of soil sampling methods used for the field projects discussed in this thesis (Chap. 2, Appendix C), findings from this study indicate that an over- or underestimation of soil NO_3-N content from a given plot would have a relatively small effect on total N fixation estimates. For example, at the Amsterdam 2010 study site (Chap. 2), a 4-core error of 8.4 kg N ha^{-1} (± 4.2 kg N ha^{-1} from plot average) in spring-planted plots terminated at pod could have introduced an error in N fixation estimates of less than 10%

for lentil and less than 5% for pea. Treatments with lower N fixation estimates would be more sensitive to error introduced by soil NO_3-N testing; however given treatment replication among blocks, error associated with individual plots would likely be reduced when averaged over treatment replicates. Future directions for this work should include multiple sites, additional sampling plots within a site, and more sophisticated spatial and statistical analyses to more completely assess the effect of sampling size on characterizing soil NO_3-N at the plot scale in a field setting.

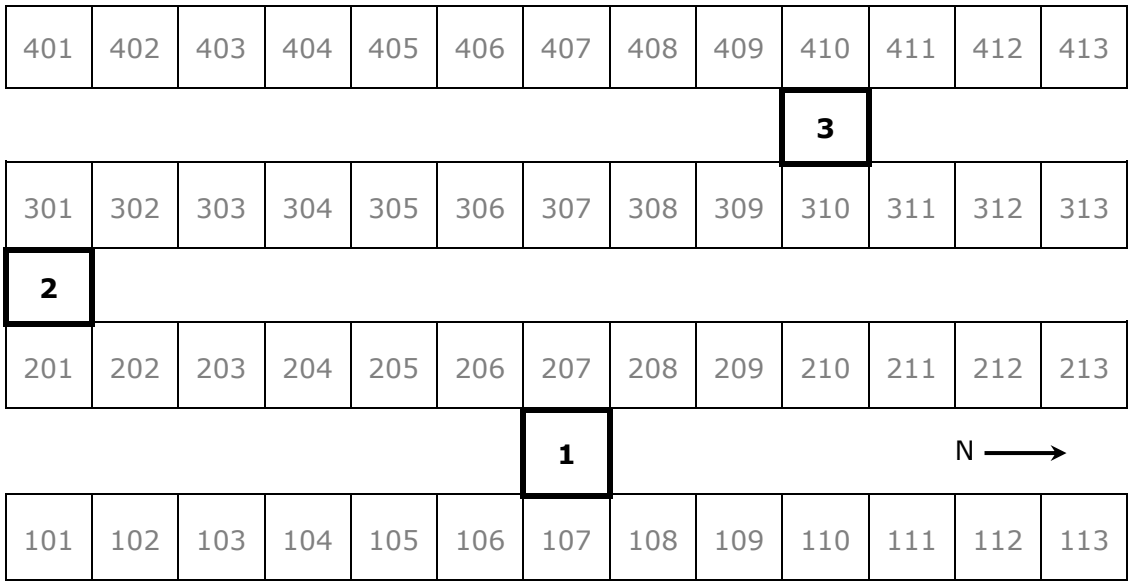

Figure F.1. Layout of sampling plots within Amsterdam (no-till green manure) 2010 study site.

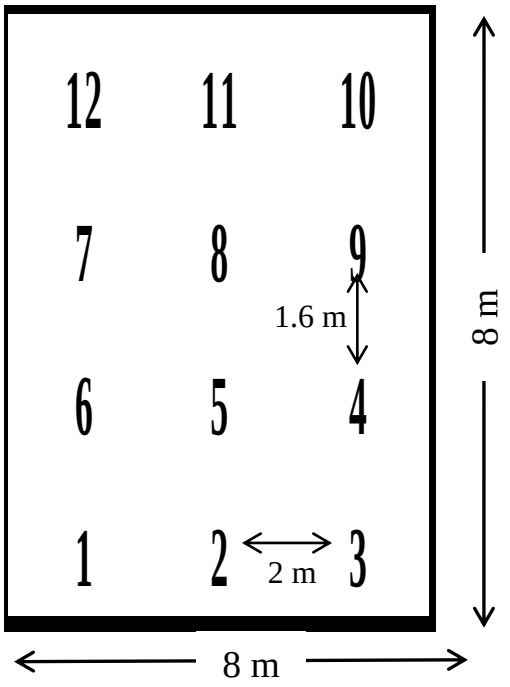

Figure F.2. Sampling locations within an 8 by 12 m plot.

Table F.1. Soil nitrate-N (kg N ha^{-1}) for the top 0.9 m soil depth at each sampling location in each plot. Sampling locations match those in Fig. F.2.

Plot 1			Plot 2			Plot 3		
33.7	49.3	46.8	45.5	56.6	49.1	42.7	43.7	16.4
48.7	56.7	54.6	61.9	39.9	36.3	43.4	36.0	32.0
41.7	36.4	40.4	73.6	48.5	41.7	29.4	22.5	28.7
21.5	22.2	23.4	53.2	45.6	45.9	42.5	53.7	59.0

Table F.2. Soil nitrate-N content (0.9-m depth) averaged (avg) from 2, 4, 6, or 12 cores from each plot. The absolute difference (diff) of each core group from the 12-core average was calculated for each plot and averaged across plots for a site average.

	Soil NO_3-N (kg N ha^{-1})						
	Plot 1		Plot 2		Plot 3		Site
No. of cores[a]	Avg	Diff	Avg	Diff	Avg	Diff	Avg Diff
2	35.8	3.9	51.1	1.3	48.7	11.2	5.45
4	31.4	8.2	48.4	1.4	40.2	2.7	4.10
6	43.1	3.5	50.3	0.5	32.0	5.5	3.17
12	39.6	--	49.8	--	37.5	--	--

[a] Sampling locations per number of cores were: 2-cores (locations 2 and 11), 4-cores (locations 1, 3, 10, and 12) and 6-cores (locations 4, 5, 6, 7, 8, and 9).

Table F.3. Mean absolute difference in soil nitrate-N (0-0.9 m depth) between core group (1, 2, 4, or 6 cores) and plot average within a plot across the study site.

No. of cores	Absolute Difference (kg N ha^{-1})
1	9.7
2	5.0
4	4.2
6	2.6

Source of variation	*df*	*P-value*
Core	3	<0.001

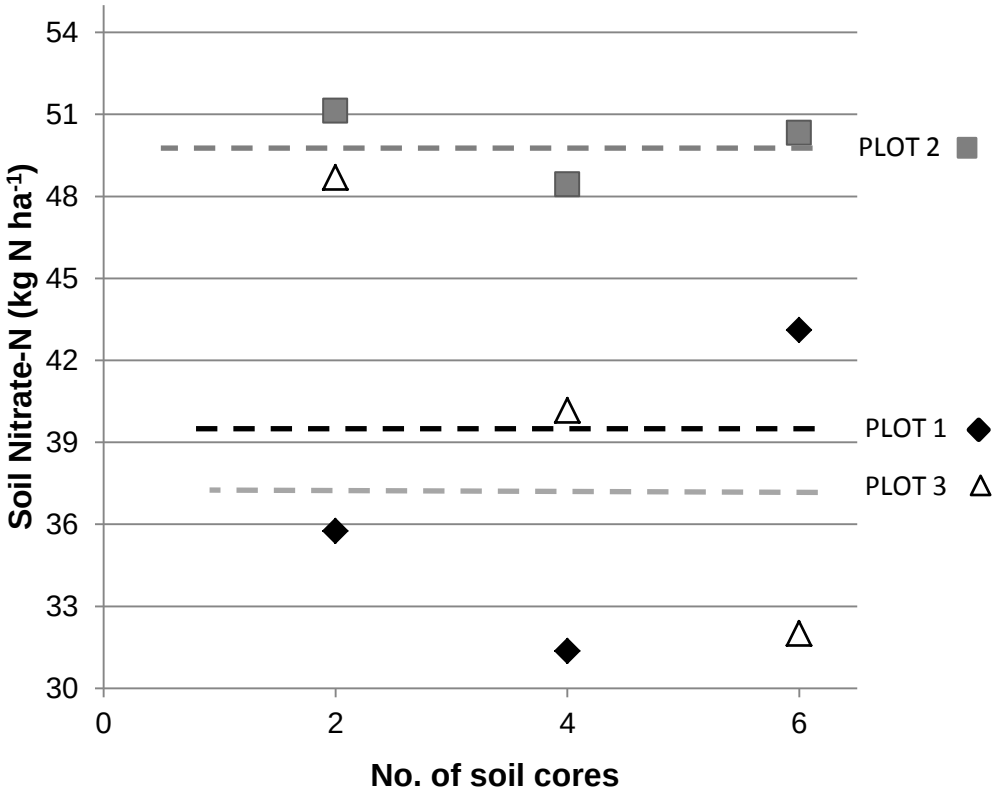

Figure F.3. Soil nitrate-N (0.9-m depth; kg N ha-1) averaged for 2, 4, 6, or 12 cores from each plot. The dashed line represents the 12-core plot average.

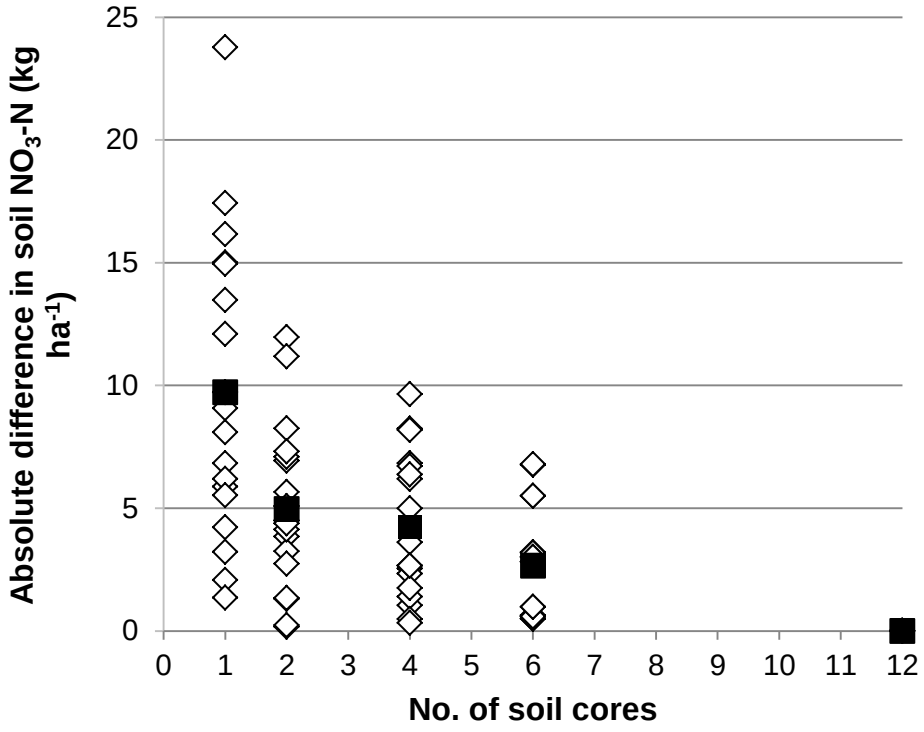

Figure F.4. Absolute difference in soil nitrate-N (0.9-m soil depth; kg N ha-1) averaged from six combinations of 1, 2, 4, or 6-cores and the 12-core plot average within a plot. The diamonds represent absolute differences for individual samples in each core group across all plots. The black squares represent the average absolute difference for each core group across all plots.