EXAMINING THE EFFECT OF ROTATION SEQUENCE ON BIOLOGICAL NITROGEN FIXATION OF PULSE CROPS

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By

Lara Renee de Moissac

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University of Saskatchewan
Room 5D34, Agriculture Building
Saskatoon, Saskatchewan
Canada, S7N 5A8

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
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ABSTRACT

Nitrogen (N) is often the most limiting nutrient in prairie crop production and is applied in the greatest quantity. Including pulse crops in rotations has become a popular option due to their ability to form symbiotic relationships with dinitrogen-fixing bacteria. This relationship means pulse crops can acquire a large proportion of their N needs from biological N fixation (BNF). In previous studies, mixed results of rotation effects on a pulse crop's ability to fix N were reported at Scott, SK, Swift Current SK, and from a greenhouse experiment using soils from Central Butte, SK. These results led to questioning if BNF is affected by a previous crop in a rotation. To address this question, research was conducted at multiple locations across Saskatchewan. The natural abundance ^{15}N isotope dilution method was used to estimate BNF in pulse crops grown on oilseed and cereal stubble in the Brown, Dark Brown, and Black soil zones. Soil samples were collected from each rotation to characterize sites and identify soil physical, chemical, and microbiological properties that may have affected BNF in pulse crops. Additionally, a controlled environment experiment was performed to determine if stubble quality (i.e., wheat and canola) affected N-mineralization potential before and after a pulse crop was grown. In the field study, an interaction between site and stubble affected BNF, where pulse crops grown on cereal stubble generally had higher BNF except at Biggar in 2017, and at Davidson and Theodore in 2018; BNF in these pulse crops was higher when grown on oilseed stubble. Inorganic N and available P contents may have affected BNF at some locations. A persistent pattern was observed in microbial biomass carbon (C) and phospholipid fatty acid (PLFA) biomarker results, where levels of each were higher in soil from pulse crops grown on oilseed stubble at Davidson, Theodore, and Springside. Climatic conditions also may have affected BNF at each location, especially in 2018, as conditions were hotter and drier compared to historical averages. In the controlled environment study, BNF was

not affected by soil or stubble; however, soil affected N acquisition and yields. A similar pattern to field results for PLFA biomarkers was observed, where total biomarkers were higher in oilseed stubbles and in the Black soil. Gross mineralization and nitrification rates were not affected by stubble before or after field pea was grown. Based on the variable results from the field and controlled environment studies, seeding pulse crops on oilseed stubble in the Brown, Dark Brown, or Black soil zones is not recommended.

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LIST OF ABBREVIATIONS

%Ndfa	Percent nitrogen derived from atmosphere
AAFC	Agriculture Agri-Food Canada
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
Ba	Barley
BG	Biggar
BNF	Biological nitrogen fixation
Cn	Canola
CB	Central Butte
CP	Chickpea
DV	Davidson
EC	Electrical conductivity
Fe	Iron
FP	Field pea
IH	Indian Head
L	Lentil
M	Mustard
MB-C	Microbial biomass carbon
MB-N	Microbial biomass nitrogen
N_2	Dinitrogen
Ndfs	Nitrogen derived from soil
NHI	Nitrogen harvest index
O	Oat
PLFA	Phospholipid fatty acid
SC	Swift Current
SP	Springside
TH	Theodore
WL	Wilkie

1 GENERAL INTRODUCTION

1.1 Introduction

Nitrogen (N) is often the most limiting factor for crop production on the prairies and continuous cropping of non-legume annual crops has become dependent on the use of synthetic N fertilizers. Rotations that include one or more pulse crop offer an alternative to traditional cereal-oilseed rotations that normally rely on synthetic N fertilizers. Pulse crops reduce the need for synthetic N fertilizer in the year they are grown because of their ability to form symbiotic relationships with N_2-fixing rhizobia bacteria. Pulse crops also supply N-rich residues to subsequent crops, thereby further reducing fertilizer inputs for the next crop and reducing the carbon footprint (Gan et al., 2011a).

Previous pulse crop research has primarily examined the benefit of N_2-fixation to a succeeding crop, not how a previous crop affects biological nitrogen fixation (BNF). Studies at Swift Current, SK and Scott, SK provide inconclusive evidence that BNF in pulse crops varies between rotations with oilseeds and cereals. At Swift Current, BNF of field pea and chickpea decreased after mustard (Knight, 2015), while at Scott, BNF in field pea increased when included in rotations with canola (Knight, 2012). In a study using soil from Central Butte, pulse crops grown on wheat stubble fixed more N from BNF than when grown on canola stubble (Chen, 2016). These results led to questioning the potential impact of a previous crop on BNF efficiency of pulse crops.

The overall goal of this research was to examine the effect of rotation on BNF. The specific objectives of this study were to: 1) determine if BNF of pulse crops is affected by the previous crop in rotation in three different soil zones in Saskatchewan; and 2) identify potential soil properties that might contribute to a preceding crop's effect on BNF. To address these objectives, field studies were completed in 2017 and 2018. Various pulse crops were grown on oilseed and

cereal stubble in field-scale situations. To address the effect of environmental conditions and soil type, eight site locations in three different soil-climatic zones across Saskatchewan were used. Additionally, a growth chamber experiment was conducted to 1) confirm field research findings under controlled environment conditions and 2) examine the capacity for microbes to provide ammonium and nitrate from preceding crop residue and potentially affect BNF in the pulse crop.

1.2 Organization of the thesis

This thesis has been prepared using a manuscript-style format. Following the General Introduction (Chapter 1) is a review of the literature (Chapter 2) focusing on BNF in pulse crops, the factors that affect BNF, and quantification techniques for estimating BNF and N mineralization rates. Chapter 3 reviews the findings from field estimates of BNF in pulse crops grown on oilseed and cereal stubble in multiple soil zones. Chapter 4 summarizes a controlled environment study that investigated the effect of two treatments, stubble type (canola and wheat) and soil type (soil from the Brown and Black soil zones) on BNF of field pea. The study also investigated the effect of the same treatments on N mineralization rates before and after field pea was grown. Chapter 5 summarizes the overall findings, discusses implications of results, and suggests future research. A compilation of the literature cited in this thesis is presented in Chapter 6 and the appendices are presented last.

2 LITERATURE REVIEW

2.1 Nitrogen use and pulse crops

Bioavailable nitrogen (N) is often the most limiting factor for crop production as it is an essential component for photosynthesis and plant growth and development (Smith, 2002; Sulieman, 2011). Synthetic N fertilizers became the major source of N for agricultural crops following World War I, establishing a new market for the ammonia produced for TNT and other explosives mass produced due to the industrialization of the Haber-Bosch process pre-World War I in 1910. The Haber-Bosch process combines N from the air with hydrogen, derived mainly from natural gas, to produce ammonia. To break the strong triple bond between N atoms and reduce an efficient amount of N into ammonia, the process requires high pressure, high temperature, the presence of a suitable catalyst and a concomitant input of energy (Gordon et al., 2001). Industrial N_2-fixation is a costly process, requiring 1% of the world's annual energy supply to produce the hydrogen gas and necessary pressure and temperatures (Smith, 2002). Production, transportation and application of synthetic N fertilizers contributes to CO_2 and N_2O emissions (Gan et al., 2011a) and due to increasing demand, the price of synthetic N fertilizer has increased dramatically over the past decades (Knight, 2012).

Through a symbiotic relationship with *Rhizobium* bacteria, pulse crops can acquire a high proportion, approximately 60% of their total N from BNF (Walley et al., 2007), thereby reducing overall synthetic N fertilizer inputs in the pulse crop phase of a rotation (Lupwayi et al., 2006; Lemke et al., 2007; Walley et al., 2007). Fertilizer N requirements are frequently reduced for a subsequent crop (Lemke et al., 2007) because pulse crops increase available soil N to the succeeding crop by adding N-rich residues to the soil (Stevenson and van Kessel, 1996a).

Rotations where lentil and field pea were included reduced effects on greenhouse gas emissions and non-renewable energy use and mineral extraction by 17 to 22% and 21 to 25%, respectively, compared to an oilseed-cereal rotation (MacWilliam et al., 2014). Additionally, pulse crops in rotation can break disease, insect and weed cycles (Krupinsky et al., 2002; Jensen et al., 2012; MacWilliam et al., 2014).

Leguminous crops grown for their dry seed are referred to as pulse crops and in western Canada include field pea (*Pisum sativum*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), and dry bean (*Phaseolus vulgaris*). Globally, pulse crops are grown for their protein-rich seeds destined for human consumption, in animals feeds, or in industrial products (Siddique et al., 2012). Studying and understanding the effects of previous crops on BNF of a pulse crop can indicate the most efficient position in a rotation of a pulse crop and improve traditional monoculture cropping systems, reduce greenhouse gas emissions, and diminish the environmental impact of agriculture (Gan et al., 2011c).

2.2 Biological nitrogen fixation

Biological nitrogen fixation (BNF) is a process where a number of species of bacteria use the enzyme nitrogenase to catalyze the conversion of atmospheric N_2 into ammonia (NH_3) (Unkovich et al., 2008). The process can be completed by free-living N_2-fixing bacteria such as Cyanobacteria, or by plant-associated bacteria symbiosis with a range of angiosperms, as is the case with *Frankia* or *Rhizobium*, where there is a significant transfer of photosynthetically fixed C from the plant to the bacteria, in exchange for biologically fixed N to the host plant. Symbiotic BNF with rhizobia in pulse crops occurs within specialized root structures of legumes called nodules (Unkovich et al., 2008).

Nodule initiation and formation by pulse crop roots in symbiosis with *Rhizobium* bacteria is a complex process. First, pulse crop roots release flavonoids and the bacterial activator protein *nodD*, signaling to rhizobia in the rhizosphere to infect root hairs of the plant (Broughton et al., 2003). Rhizobia react to this signal molecule by inducing the genes responsible for nodulation (Gage, 2009) and in turn emit host-specific Nod factors that induce root-hair deformation in the plant (Broughton et al., 2003; Lupwayi et al., 2006). Plant host and bacteria signal recognition contributes to the specificity of these interactions (Gage, 2009). The Nod factors secreted are lipochitooligosaccharides that contain species-specific end substitutions responsible for subsequent steps in the signal transduction of BNF (D'Haeze and Holsters, 2002; Geurts et al., 2005). Close contact between rhizobia and the root hair must be established in order for attachment to occur (Lie, 1981). Root hairs grow around the bacteria, trapping it between root hair cell walls (Gage and Margolin, 2000; Hirsch et al., 2001; Broughton et al., 2003). Encased bacteria continue to grow, forming a micro-colony, and continue secreting Nod factor, which causes the cell walls to reorient inwards, forming a tunnel or infection thread, instead of growing outwards like a normal root hair (Gage, 2009). Cortical cell division of the root starts at the same time as infection thread formation, giving rise to nodule primordium and meristem development (Lupwayi et al., 2006). Rhizobia bacteria then infect the nodule through the infection thread and enter the cytoplasm of the nodule cells (Oke and Long, 1999). Rhizobia are protected within the nodule cell cytoplasm by a peri bacteroid membrane that provides physical protection against host cell defense reactions and controls nutrient exchange between the symbiotic partners (Lupwayi et al., 2006).

Rhizobia in colonized nodules differentiate into a distinct cell-type called a bacteroid, that is capable of fixing N_2 (Oke and Long, 1999). Using the enzyme nitrogenase and energy from photosynthesis, rhizobia transform adenosine tri-phosphate (ATP) into adenosine di-phosphate to

break the strong triple bond in dinitrogen (N_2) (Havlin et al., 2005; Strodtman and Emerich, 2009) thus, catalyzing the conversion of N_2 into ammonia. Nitrogenase has a short half-life in the presence of oxygen, yet bacteroids require oxygen to complete the N_2-fixation process. Leghemoglobin carries oxygen from outside the nodule through the nodule cell cytoplasm to the bacteroids (Strodtman and Emerich, 2009). Leghemoglobin controls the concentration of oxygen in the nodule and balances the protection of nitrogenase against oxygen with respiration functions (Nap and Bisseling, 1990). The presence of leghemoglobin indicates an active nodule and is pink or red in colour (Sylvia et al., 1998) due to the oxidation of ferrous leghemoglobin to ferric leghemoglobin (Becana and Klucas, 1990). The result of the nitrogen fixation process is useable ammonium ions for the production of proteins by the plant (Havlin et al., 2005). Most ammonium is used by the host plant, but some may be excreted from the nodule, used by other plants, or released when nodules die and decompose (Havlin et al., 2005).

2.3 Factors that affect biological nitrogen fixation

2.3.1 Crop rotation

The current recommendation for including a pulse crop in rotation is every three to five years (Hnatowich, 2000; Malhi et al., 2011). Depending on a pulse crop's position within a rotation, yield or BNF may be affected. A study at Swift Current, SK found that pulse crops grown immediately after canola produced less biomass and BNF was lower compared to pulse crops grown after wheat (Chen, 2016). A study conducted at Scott, SK reported cumulative seed yield of pea was usually lower in monoculture pea compared to when pea was included every two or more years, i.e. pea-wheat, pea-canola-wheat or canola-wheat-pea-wheat (Malhi et al., 2011). In another study using the same experimental plots at Scott, SK, Knight (2012) reported that N uptake

and productivity parameters for field pea were affected by rotation. The percentage of N derived from atmosphere obtained from BNF in continuous pea in 2008 was 18% and in 2009, 14% but was not due to a lack of precipitation, nutrient supply, or disease in any year. In contrast, pea grown in pea-wheat-canola-wheat and pea-canola-wheat rotations derived 50 to 59% of their N from BNF (Knight, 2012). Differences in BNF from this study were not due to concentrations of inorganic N in the continuous pea rotation inhibiting BNF or due to disease.

2.3.2 Soil microbial community

Agroecosystem management interventions such as tillage, crop species composition and soil amendments act in concert with the background soil environment to alter indigenous soil biota and to influence rhizosphere community composition (Buckley and Schmidt, 2003; Drinkwater and Snapp, 2007a). The influence of crop species composition is most significant for plant-associated habitats such as the rhizosphere and rhizoplane (Salles et al., 2004). Plant roots play an important role in shaping microbial communities in soil by releasing a wide range of compounds (Salles et al., 2004) known as root exudates. Some substances released into the rhizosphere such as root exudates, sloughed root cells and mucilages can serve as C or N sources for bacteria and fungi (Buyer et al., 2002) and support the metabolic activities of diverse groups of microorganisms (Bais et al., 2006). The amount and kind of root exudates differ between plant species, and these differences can stimulate species-specific shifts in the soil microbial community (Lupwayi et al., 1998; Welbaum et al., 2004). Legume exudates differ in amount and composition from those of other crops species, and therefore may impact the rhizosphere community (Ibekwe and Kennedy, 1998).

In addition to crop root exudates, previous crop residue can affect soil microbial composition as the rate of decay and the amount of nutrient released to soil may depend on crop

species (Gan et al., 2011b). Chemical changes in soil mediated by crop rotations are caused predominantly by the build-up of root exudates and residues from preceding crops (Garbeva et al., 2004). Chemical properties of crop residues such as C:N ratio, N concentration, lignin, and/or polyphenol concentration can affect residue decomposition rate (Vigil and Kissel, 1991; Wang et al., 2004). The decomposition rate of field pea, canola, and wheat straw was directly related to residue N concentration and/or C:N ratio (Janzen and Kucey, 1988) and lignin content played only a secondary role, if any (Soon and Arshad, 2002). Functional groups within a soil microbial community may favour, or are effective at, degrading different parts of organic matter (OM). Actinobacteria are effective at degrading complex organic materials including cellulose and lignin (de Boer et al., 2005). Similarly, fungi are important in the degradation of lignocellulosic materials contained in crop residues (McMahon et al., 2005; Schneider et al., 2012). Gram positive bacteria are presumed to be adapted to more complex and partially decomposed litter, due to their relatively slow growth habit (Rubino et al., 2010).

By including field pea with canola and wheat in various rotational combinations, microbial biodiversity may be increased, making the microbial community more flexible and able to respond to environmental or biotic fluctuations that affect BNF (Knight, 2012). At Indian Head, SK, microbial communities were smaller under a continuous pea rotation compared to a pea-wheat rotation; crop productivity, soil OM level and microbial community structure and function were negatively affected in the continuous pea rotation (Nayyar et al., 2009).

2.3.3 Inoculation

2.3.3.1 Indigenous rhizobia versus commercial inoculant

The rhizobia-host plant relationship is species-specific and therefore crop variety and rhizobial strain can both affect BNF (Yang et al., 2017). For field pea, lentil and faba bean, *Rhizobium leguminosarum* is the specific species required. For chickpea, the necessary species is *Rhizobium cicer* and for soybean, *Bradyrhizobium japonicum* (Somasegaran and Hoben, 1994). A study using topsoil with optimized nutrient contents found that nodule numbers on crown and lateral roots of field pea differed two-fold when inoculated with two different *R. leguminosarum* bv viciae strains (Yang et al., 2017). The amount of fixed N in shoot and root tissues differed by 1.5 to 2-fold, confirming that BNF capabilities of legume plants differ when inoculated with different rhizobia strains (Yang et al., 2017).

Competition among strains of rhizobia for pulse crop nodulation is a major practical problem that frequently results in highly effective N_2-fixing strains becoming ineffective in the field because they are out-competed by better-adapted, but usually less effective, indigenous strains (Lupwayi et al., 2006). Due to the cost of strain evaluation for commercial inoculants, effectiveness is the main criterion (Lupwayi et al., 2006). Commercial inoculant application increased total dry matter, total N and BNF in lentil, field pea and faba bean under dryland conditions (Bremer et al., 1988). By applying a commercial inoculant at seeding, the appropriate strain of rhizobia is supplied in a sufficient quantity to support BNF (Hardarson and Atkins, 2003).

Commercial inoculants can be formulated from a single *Rhizobium* strain or multiple strains, depending on the pulse crop to be inoculated. Two *R. leguminosarum* strains are often combined into one inoculant for application on field pea or lentil (Hnatowich, 2000) increasing the versatility of the inoculant.

2.3.3.2 Inoculant application

Commercial inoculants are applied directly to seed or soil in a carrier, such as powdered peat, granular clay, or liquid. The application of commercial inoculants can affect the location of nodules on roots. While the most efficient method of applying inoculants is by coating the seed, either with a peat carrier or liquid formulation, application on or with the seed often results in predominantly root crown or tap root nodules forming (Rice et al., 2000; Hardarson and Atkins, 2003). Alternatively, soil-applied granular inoculant induces formation of nodules throughout the root system including around root crowns and on lateral roots (Rice et al., 2000; Hynes et al., 2001). A study at Fort Vermillion, AB and Beaverlodge, AB found that soil-applied inoculant on field pea increased nodule number and biomass N accumulation at flat-pod by 28-90%, total N accumulation by 21 to 55% and BNF by 130 to 305% compared with field pea with seed-applied inoculant (Clayton et al., 2004). Nodules formed on lateral roots provide the majority of fixed N during the later stages of plant growth (Hardarson et al., 1989). Furthermore, while crown nodules form earlier than lateral nodules, they also senesce earlier, indicating that the younger lateral nodules may be more useful to plants during later nutrient demanding reproductive stages (van Kessel, 1994; Rice et al., 2000).

2.3.4 Nutrient management

2.3.4.1 Inorganic N

In addition to using superior *Rhizobium* strains, any practice that increases the N demand of the host plant should increase BNF in pulse crops (van Kessel and Hartley, 2000). However, when soil N is in sufficient supply to meet the demand of the crop, even the most effective rhizobia-host plant relationship will result in little BNF (van Kessel and Hartley, 2000). Excess nitrate

availability reduces nitrogenase activity because of an increase in competition between nitrate reduction processes and BNF reactions within the plant, therefore reducing BNF (Havlin et al., 2005). Furthermore, the decomposition of OM in soil, and the accompanying mineralization of organic N into ammonium and nitrate which may contribute to competition within the host plant, may vary depending on the quantity of mineralizable N and environmental conditions, soil moisture and temperature, that control the rates of the process (Watkins and Barraclough, 1996; Curtin and Campbell, 2002).

2.3.4.2 Phosphorus, potassium, and sulfur

Pulse crops that acquire N through BNF generally have a higher requirement for phosphorus (P), potassium (K), and sulfur (S) than those that rely on soil N alone (Israel, 1987; Sulieman et al., 2013). Proper P, K, and S supply support aspects of BNF in legumes such as nodule activity, nodule growth and function, and nitrogenase activity. An alleviation in P deficiency in soybeans dependent on BNF alone caused increases in nodule mass per plant, nodule number per plant, and average mass per nodule (Israel, 1987). High rates of potassium-chloride and potassium-sulfate fertilizer applied to alfalfa increased nodule number and nitrogenase activity, compared to control plants in soil from a K-deficient region of the mid-west U.S. (Duke et al., 1980). There is a close relationship between S supply and nitrogenase and leghaemoglobin content in nodules (Scherer et al., 2008; Varin et al., 2010). Under S starvation, BNF is reduced as a consequence of reduced leghemoglobin concentration as well as reduced ATP supply (Scherer et al., 2008).

2.3.4.3 Other nutrients

Micronutrients are generally required for plant growth and development processes, but a few micronutrients play particularly important roles during BNF (Bonilla and Bolaños, 2009). Iron

(Fe) and molybdenum (Mo) are essential micronutrients because they are part of two essential metalloproteins that are the principal components of nitrogenase: the Fe protein and the MoFe protein (Smith, 2002). Within a nodule, Fe is also important for heme-containing proteins such as leghemoglobin and Fe-S proteins such as ferredoxin (Bonilla and Bolaños, 2009).

Nodulation and BNF in legume-*Rhizobium* symbioses are dependent on Boron (B) and Calcium (Ca^{2+}). Under normal plant growth or abnormal stress conditions, a relationship between B and Ca^{2+} exists. Boron is essential for nod gene induction, root hair curling, and adsorption of bacteria to the root surface, while Ca^{2+} enhances cell and tissue invasion by *Rhizobium*, which are highly impaired by B deficiency (Bonilla and Bolaños, 2009). Independently, B is essential for synthesis and stability of cell walls and when deficient, affects infection thread formation (Bolaños et al., 1994). Infection threads in B-deficient legumes are extremely enlarged and abort prior to bacterial release (Bolaños et al., 1996). Development of infection threads was diminished by about 30% in B-deficient plants (Redondo-Nieto et al., 2001). Three- and four-week-old B-deprived nodules showed degeneration of cell walls and membranes and appeared white, showing no signs of nitrogenase activity, confirming the essential role of B in nodule development (Bolaños et al., 1994, 1996). Calcium is responsible for cell wall integrity of free-living *Rhizobium* (O'Hara et al., 1988) and for optimal root hair colonization (Lodeiro et al., 1995). Attachment of rhizobia is mediated by plant and bacterial components able to use Ca^{2+} to reinforce adhesion between rhizobia and roots (Bonilla and Bolaños, 2009).

2.3.5 Soil pH

Soil pH affects the survival, growth and BNF of rhizobia, while nutritional disorders affect the symbiotic relationship between host plant and rhizobia (Lie, 1981; Tang and Thomson, 1996).

Rhizobia implement strategies to maintain intracellular pH such as decreasing membrane permeability, internal buffering, amelioration of external pH, proton extrusion/uptake and prevention of metal ion toxicity (Dilworth and Glenn, 1999). Following a pH change, species-dependent genes are triggered to facilitate cell function adaptations in order for the bacteria to survive and grow (Hirsch, 2010). Fewer genes have been found that are induced under alkaline conditions partly due to fewer studies examining alkaline tolerance (Hirsch, 2010).

Optimal soil pH for pulse crop growth is neutral or slightly acidic (Bordeleau and Prevost, 1994). The effects of pH on growth and nodulation of 14 grain legume species, including field pea, lentil, chickpea, and faba bean, grown in nutrient solution, supplied with N or solely reliant on BNF, showed tolerance to a range of pH, but intolerance to extremes of pH as indicated by shoot growth, nodule numbers and nodule mass (Tang and Thomson, 1996). Lentil was the most sensitive to acidic conditions while field pea, chickpea and faba bean were intolerant to high or low pH (Tang and Thomson, 1996).

In a study using acidic soils from Peace River, AB, soil pH affected biomass, pink nodule formation and active nodule weight in field pea (Rice et al., 2000). Active nodule number and weight increased linearly as soil pH increased from 4.4 to 6.6. Biomass increased as pH increased from 4.4 to 5.4 but decreased as pH further increased from 5.4 to 6.6 (Rice et al., 2000). This study also reported that granular inoculant was effective for establishing nodules at soil pH 4.4, granular and powdered peat were effective at pH 4.4 and 5.4, and granular, powdered peat, and liquid inoculants were effective at pH 6.6. Using the same soils, a local isolate of *R. leguminosarum* bv *viciae* was used to inoculate field pea and established nodules at pH 6.6, but like the commercial liquid formulation, failed to establish effective nodules at pH 4.4 and 5.4 (Rice et al., 2000).

Growth inhibition and reduced survivability of rhizobia in acidic soils is caused by increased hydrogen ion concentration, and increased solubility of the toxic metal ions aluminum (Al^{3+}), copper (Cu^{2+}) and manganese (Mn^{2+}) that both affect intercellular pH stability (Graham et al., 1994). Soil acidity disrupts the signal exchange between the host plant and rhizobia starting with reduced flavonoid secretion by the plant (Hungria and Stacey, 1997). The reduction in flavonoid secretion decreases rhizobia Nod gene induction and restricts Nod factor and Nod metabolite excretion (McKay and Djordjevic, 1993), which in turn affects the chain of events leading to root hair deformation and curling (Miransari et al., 2006). Root growth of the host plant is hindered in acidic conditions. In acidic soils, Al^{3+} accumulates at root apices causing physical damage and build-up to toxic levels inhibits root cell division and elongation (Ryan et al., 1993), decreasing macronutrient availability (Ferguson et al., 2013).

2.3.6 Environmental conditions

2.3.6.1 Temperature

Temperature can affect rhizobia survival in soil and can limit both nodulation and BNF (Graham, 1992; Chalk et al., 2010). Temperature is a primary determinant affecting host plant metabolic processes such as rates of respiration, photosynthesis, transport and transpiration (Bordeleau and Prevost, 1994). For most rhizobia the optimum temperature for growth in culture is from 28 to 31°C, with many unable to grow below 10 or above 37°C (Graham, 1992). The optimum temperature range for photosynthesis of legumes is between 15 and 25°C (Lie, 1981) and at higher temperatures, photosynthesis is drastically reduced (Bordeleau and Prevost, 1994). A delay in onset of nodulation and decrease in nodule number, size and growth rate occurred in bean,

field pea and lentil grown in water baths, when temperature was outside the optimal range (Lira Junior et al., 2005).

The root system depends on the shoot for the supply of carbohydrates from photosynthesis (Lie, 1981). Root respiration may use up to 50% of photosynthates produced in one day (Bordeleau and Prevost, 1994). If temperature affects photosynthesis and respiration, and in turn plant growth and productivity, it will also affect BNF because both of these processes determine carbohydrate availability for BNF (Whittington et al., 2012). When four different native perennial legume species were grown at 25°C and 28°C, relying on BNF alone, 100% of *Lupinus* seedlings had nodules at 25°C, while only 40% of seedlings had nodules at 28°C (Whittington et al., 2012).

2.3.6.2 Topography and water use

Biological nitrogen fixation is strongly influenced by topography, which controls the distribution of water and pedalogic processes (Stevenson and van Kessel, 1996b; van Kessel and Hartley, 2000). Legumes grown in depressions can either exhibit increased or decreased BNF compared to plants grown in non-depression positions (Stevenson et al., 1995). Even though soil water is essential for optimal BNF, an excess of water will reduce nitrogenase activity because of a lack of oxygen (Sprent, 1972). Biological nitrogen fixation declined concurrently with a decline in water use by lentil, pea and faba bean showing that plant water use and BNF are connected (Bremer et al., 1988). If topography strongly influences the distribution of water, pulse crops are ideally grown on level to gently undulating fields.

Pulse crops are well-adapted to a range of water regimes. The shallow roots of pulse crops in comparison to wheat or canola root structures allows them to use less water while still maintaining adequate yields (Angadi et al., 2008). Under irrigated, rainfed and imposed drought conditions, field pea used the least amount of water and had the highest grain yield, and desi

chickpea used water as availability increased and had intermediate yield (Angadi et al., 2008). Under irrigated conditions, lentil had the lowest water use efficiency and yield (Angadi et al., 2008) suggesting its suitability for drier conditions. A study in south-western Australia with comparable rainfall conditions to south-western Saskatchewan, (i.e., < 350 mm yr^{-1} compared to an average 351 mm yr^{-1}, respectively) showed faba bean yield and growth to be variable (Mwanamwenge et al., 1998) suggesting it is more suited to climatic zones with higher average precipitation.

2.4 Quantification of biological nitrogen fixation

2.4.1 Nodulation assessment

Nodule number and weight are often positively correlated with BNF and can be useful measurements to help interpret data collected from other measurements (Hardarson and Danso, 1993). A nodulation health assessment takes into account plant growth and vigour, colour and abundance of nodules, and nodule positions on a root system (Cardoso et al., 2009). Additionally, a visual appraisal of red pigmentation from leghemoglobin content provides a relative measure of a nodule's effectiveness (Hardarson and Atkins, 2003).

2.4.2 Isotope dilution techniques

To maximize the benefits of the pulse crop-rhizobia symbiosis, it is useful to estimate the amount of N fixed under field conditions (Hardarson and Danso, 1993). One of the most common methods to estimate BNF is the isotope dilution method. The ^{15}N-isotope dilution method measures uptake of ^{15}N in an N_2-fixing crop comparing it to a reference crop or plant that does not fix N_2 (van Kessel and Hartley, 2000). There are two ways to measure BNF using this method,

either through enrichment with ^{15}N-labeled fertilizer or by taking advantage of differences in natural ^{15}N abundance levels. Erroneous estimates of BNF can occur because of differences in seasonal N accumulation patterns of legume and the reference crop under field conditions, the concurrent decline in atom% ^{15}N of the available soil N pool or differences in root distribution (van Kessel and Hartley, 2000). Use of different non-N_2-fixing reference crops can also lead to widely variable estimates in BNF (Witty, 1983; Danso, et al., 1993).

2.4.2.1 Enriched isotope dilution method

In the enriched isotope dilution method both N_2-fixing and non-N_2-fixing crops are grown in soil where the same amount of ^{15}N-labeled fertilizer has been applied (Hardarson and Danso, 1993). In the absence of N-supply other than soil and ^{15}N-labeled fertilizer, both plants will contain the same ratio of ^{15}N to ^{14}N, since they are taking up N of similar ^{15}N to ^{14}N composition, but not necessarily the same total quantity of N (Hardarson and Danso, 1993). With BNF, the N_2-fixing plant will contain a lower ratio of ^{15}N to ^{14}N due to incorporation of atmospheric N_2 which has a lower ^{15}N to ^{14}N ratio than soil and the applied fertilizer (Hardarson and Danso, 1993). Harvested plant materials are analyzed for ^{15}N content and the dilution of ^{15}N-labeled fertilizer by ^{14}N derived from atmospheric N_2 compared to the same contents in the reference plant and the proportion of N fixed from atmosphere are calculated (Unkovich and Pate, 2000).

2.4.2.2 Natural abundance method

As a result of isotope discrimination effects that occur during soil formation, most soils have a slightly higher ^{15}N abundance than the atmosphere (Hardarson and Danso, 1993). Similar to the EN technique, the natural abundance method relies on a significant difference in the ratio of ^{15}N to ^{14}N between atmospheric N_2 and the pools of soil N that the N_2-fixing and non-N_2-fixing plants are utilizing in a field situation (Unkovich et al., 1994). Nitrogen fixing plants may have a lower

^{15}N enrichment than non-fixing ones and therefore, the NA technique is another useful method to measure BNF (Amarger et al., 1979; Kohl et al., 1980; Hardarson and Danso, 1993). An advantage of the natural abundance technique is that there is no requirement to add ^{15}N-labeled fertilizer, making it suitable for field-scale studies (Chalk, 1985). However, the precision of the natural abundance technique becomes questionable when the difference in ^{15}N abundance between the soil and atmosphere is small (Unkovich et al., 1994) or when there is large spatial variability in natural ^{15}N abundance (Holdensen et al., 2007).

2.5 Determining N mineralization rates using isotope pool dilution

Microorganisms in soil are responsible for N and C cycling and usually draw available N in proportion to that of available C (Bengtsson et al., 2003) making C:N ratios of a crop an important factor for nutrient cycling by microorganisms. At any time, microbes are decomposing organic matter (OM) with narrow or wide C:N ratios resulting in mineralized organic N or immobilized N, depending on the C:N ratio (Powlson and Barraclough, 1993).

Isotope pool dilution allows the determination of gross rates of mineralization and nitrification in the presence of decomposing crop residues, unconfounded by processes consuming ammonium (NH_4^+) (Watkins and Barraclough, 1996). Briefly, the isotope pool dilution technique is as follows. When the soil NH_4^+ pool is at time equals zero (t = 0), a quantity of labeled NH_4^+ is added. The tracer is diluted as a consequence of mineralization of unlabeled organic N to NH_4^+ (Braun et al., 2018). Over time the amount of unlabeled NH_4^+ will increase, and the proportion of labeled NH_4^+ will decrease. Gross N mineralization is then calculated from the change in size of the total NH_4^+ pool (^{15}N + ^{14}N) and from the decline in the ^{15}N enrichment above natural abundance (Kirkham and Bartholomew, 1954; Hart et al., 1994; Murphy et al., 2003). Processes that consume

NH_4^+ will remove labeled or unlabeled NH_4^+ from the pool in proportion to the amounts present under two conditions: if the added label mixes with the indigenous soil NH_4^+; and if no preferential utilization of either labeled or unlabeled NH_4^+ occurs (Powlson and Barraclough, 1993). If these two assumptions are met, the processes that consume NH_4^+ will not in themselves alter the ^{15}N abundance in the pool. The principles and assumptions of the process are the same for nitrate (NO_3^-) production through nitrification. Methodological considerations that must be determined prior to the start of the experiment include uniformity of tracer distribution in the soil, changes in soil conditions as a result of labelling, eliminating the potential for ^{15}N tracer losses either through leaching or volatilization, and the optimum duration of the experiment (Di et al., 2000).

3 EFFECT OF ROTATION ON BIOLOGICAL NITROGEN FIXATION OF PULSE CROPS

3.1 Preface

This chapter examines the effect of rotation sequence on biological nitrogen fixation of pulse crops after oilseed or cereals at sites in the Brown, Dark Brown and Black soil zones. This chapter focuses on BNF and N acquisition as well as soil physical, chemical, and microbiological properties that may affect BNF. The results obtained provide information on whether a certain rotation should be avoided. The results also assist in optimizing BNF efficiency in rotation with the potential to assist producers in minimizing synthetic fertilizer use in prairie agriculture systems.

3.2 Abstract

Pulse crops are an attractive option for producers to include in rotation because of their ability to biological fix N_2 (BNF) in symbiosis with rhizobia bacteria. Prior research has focused on subsequent N benefit of pulse crops, not on the effects of a preceding crop on BNF. Research was conducted at multiple locations across Saskatchewan in 2017 and 2018. The natural abundance ^{15}N isotope dilution technique was used to estimate BNF in pulse crops grown on oilseed and cereal stubble in the Brown, Dark, Brown and Black soil zones. Soil samples were collected from each rotation to characterize sites and identify potential soil properties that may have affected BNF. In both years, BNF was affected by an interaction between site and stubble, where pulse crops grown on cereal stubble had higher BNF except at Biggar in 2017, and Davidson and Theodore in 2018, where BNF was higher on oilseed stubble. Levels of inorganic N and P may have affected BNF at some locations. A persistent pattern was observed in microbial biomass

C and PLFA biomarker results, where levels of each were higher in soil from pulse crops grown on oilseed stubble at Davidson and Theodore. Weather conditions also may have affected BNF at each location, especially in 2018, when conditions were hotter and drier compared to historical averages.

3.3 Introduction

Continuous cropping of non-legume crops such as cereals and oilseeds has become dependent on the use of synthetic N fertilizers. An alternative to the traditional cereal-oilseed rotations is to include a pulse crop in a rotation. Pulse crops may reduce the reliance on synthetic N fertilizers because of their ability to fix N in symbiosis with N_2-fixing bacteria. Pulse crops may also further decrease the need for synthetic N fertilizer through their addition of N-rich residues that become available to the succeeding crop (Gan et al., 2010).

Pulse crop research in the past has primarily examined the contribution of N to succeeding crops, not how a previous crop may affect BNF. Inconclusive evidence found that BNF in pulse crops varied between rotations with oilseeds and cereals at different locations in Saskatchewan. At Swift Current, BNF of field pea and chickpea decreased after mustard (Knight, 2015), at Scott, BNF in field pea increased in rotations that included canola (Knight, 2012), and at Central Butte, pulse crops grown on wheat stubble fixed more N from BNF than those grown on canola stubble (Chen, 2016). Based on these results it was hypothesized that BNF of pulse crops would be affected when in rotation with cereals or oilseeds.

The first objective of the field experiment was to estimate BNF in pulse crops grown after cereals and oilseeds and determine if cropping sequence does in fact affect BNF. The second objective was to characterize each site and relate soil physical, chemical, and microbiological

properties that might contribute to a preceding crop's effect on BNF. In addition to estimates of BNF measured by percent N derived from atmosphere (%Ndfa), measurements of aboveground N (ABG-N), fixed N, N derived from soil (Ndfs), and nitrogen harvest index (NHI) were calculated. These measurements may assist in estimating the contribution of N by pulse crops to a succeeding crop as affected by rotation (Knight, 2012; Xie et al., 2017).

3.4 Materials and Methods

3.4.1 Site descriptions and experimental design

Field studies were conducted in the 2017 and 2018 growing seasons at a total of eight sites in Saskatchewan. The locations chosen were representative of typical pulse crop growing regions in the Brown, Dark Brown and Black soil zones and are defined by the town or city, closest to where the research was conducted. Sites are specified as the exact location where the research was conducted. In 2017, sites were located near Biggar (BG) and Wilkie (WL) and the experimental treatments included site and stubble type (Table 3.1). In 2018, sites were located near Biggar, Central Butte (CB), Davidson (DV), Theodore (TH), and Springside (SP). At each location a minimum of two fields were selected: one with a pulse crop (chickpea, field pea or lentil) growing on a cereal (wheat, oat or barley) stubble; one with the same pulse crop species growing on a brassica (either canola or mustard) stubble. Non-N_2-fixing weeds from each field were sampled for calculating %N derived from atmosphere (%Ndfa). Research plots with the same crop sequences at Agriculture and Agri-Food Canada (AAFC) at Indian Head (IH) and Swift Current (SC) were also sampled in 2018. Experimental treatments included site and stubble type (Table 3.1). In 2018, at Swift Current and Biggar, an additional species of pulse crop grown on oilseed and cereal stubbles was sampled (Table 3.1). One corner of the plot area at Indian Head had known

salinity issues so this area was avoided for this study's experimental set-up. The plots were also quite weedy which may have affected plant growth through competition for moisture and nutrients. Fields and plots were seeded and managed by producers and AAFC staff, respectively. All pulse crops were inoculated with a commercial *Rhizobium* inoculant. Close proximity, ideally less than 2 km between pulse crop and non-N_2-fixing reference crop fields, ensured soil properties and growing conditions were similar.

Table 3.1. Locations and crop sequences for the 2017 and 2018 field experiments.

Location	Soil Zone	Sequences			
		Oilseed 1	Cereal 1	Oilseed 2	Cereal 2
		2017			
Biggar	Dark Brown	Cn†-FP	B-FP	-	-
Wilkie	Dark Brown	Cn-FP	W-FP	-	-
		2018			
Central Butte	Brown	Cn-FP	W-FP	-	-
Swift Current	Brown	M-CP	W-CP	M-L	W-L
Biggar	Dark Brown	Cn-FP	W-FP	Cn-L	W-L
Davidson	Dark Brown	Cn-L	W-L	-	-
Indian Head	Black	Cn-FP	W-FP	-	-
Springside	Black	Cn-FP	O-FP	-	-
Theodore	Black	Cn-FP	O-FP	-	-

† CP = chickpea, FP = field pea, L = lentil, Cn = canola, M = mustard, B = barley, O = oat, and W = wheat

3.4.2 Weather data

Weather data was collected from Environment Canada weather stations nearest to the eight sites (Environment Canada, 2011). Mean daily maximum temperatures and cumulative monthly precipitations for the growing season (May, June, July and August) of each year were compared to the historical data (1981-2010).

3.4.3 Soil sampling and analyses

Soil sampling was conducted in 2017 and 2018 at each site in late April or early May before seeding. Soil cores were taken from each field from depths of 0 to 15 cm and 15 to 30 cm using a Dutch auger (3 cm dia.). Samples were taken randomly across the plot area, avoiding seed rows, saline areas, or field approaches where compaction could have occurred. From producer's fields 10 samples were collected from each depth and combined into one composite sample for each depth per field. At the AAFC sites at Indian Head and Swift Current, two cores were taken from the middle of each plot, approximately 3 m apart. Cores were combined into one sample for each depth and two samples per plot. After mixing to homogenize the composite sample, a subsample of moist field soil was taken and stored at 4 °C for inorganic N analysis. The remainder of the composite sample was air dried and ground to pass a 2-mm sieve, using a particle size soil grinder (Humboldt Manufacturing, Elgin, IL, USA).

3.4.4 Soil pH, electrical conductivity, and texture

For each sample, 40 g air-dried soil:80 mL deionized water was used to measure pH and EC (Hendershot et al., 2007; Miller and Curtin, 2007) on a calibrated pH and EC meter (PC700, Oakton, ON, Canada). Particle size analysis was determined using the Bouyoucos hydrometer method (Thien and Graveel, 2008), adjusting the second reading time from 2 h to 6 h to remove bias in % clay associated with a 2 h reading (Ashworth et al., 2001).

3.4.5 Total nitrogen and organic carbon

Soil samples were air dried then ball ground until they were able to pass a 0.075 mm sieve. Total N content was analyzed on a LECO TruMac CNS Analyzer (LECO Corporation, St. Joseph, MI, USA). For organic C analysis, carbonates were removed from 1 g subsamples by first weighing soil into nickel-lined ceramic combustion boats and placing on a 70 °C hot plate (Skjemstad and Baldock, 2007). Samples were moistened with distilled water then acidified with 6% (w/v)

sulfurous acid to remove inorganic carbonates. When samples stopped reacting (forming CO_2), an additional 1 mL of sulfurous acid was added, then samples were allowed to dry (Skjemstad and Baldock, 2007). Organic C was measured using a LECO C632 (LECO Corporation, St. Joseph, MI, USA).

3.4.6 Inorganic nitrogen, available phosphorus, potassium, and sulfur

Inorganic N was extracted from field-moist soil subsamples using 2M KCl (Maynard et al., 2008). Briefly, 5 g of field-moist soil was weighed into disposable vials and 50 mL of 2M KCl solution added. Subsamples were shaken at 142 rpm for 1 h, then filtered through Whatman No. 42 filter paper (Whatman, Maidstone, UK) into clean vials. Samples were frozen until analysis on a Technicon Autoanalyzer (SEAL Analytical, Mequon, WI).

Available phosphorus (P) and potassium (K^+) were extracted using the Modified Kelowna extraction method (Qian et al., 1994). Modified Kelowna solution was prepared with 1.4 % (w/w) acetic acid, 1.9 % (w/w) ammonium acetate, and 0.056 % (w/w) ammonium fluoride in distilled water. Into disposable vials, 3 g of air-dried soil and 30 mL of Kelowna solution were added, then capped and shaken on a rotary shaker at 142 rpm for 5 min. Soil suspension was filtered through Whatman No. 42 filter paper into clean vials. Phosphorus extracts were analyzed on a Technicon Autoanalyzer (SEAL Analytical, Mequon, WI) and K^+ extracts were analyzed on an Agilent Atomic Absorption Flame Emission Spectrometer for 2017 samples and an Agilent Microwave Induced Plasma Spectrometer for 2018 samples (Agilent Technologies, Santa Clara, CA, USA).

Sulfate was extracted with 0.01M $CaCl_2$ (Houba et al., 2000). Calcium chloride solution was prepared by dissolving 1.11 g $CaCl_2$ into 1 L of distilled water. Twenty g of air-dried soil was weighed into extraction bottles and 40 mL of extraction solution was added. Samples were capped and shaken on a rotary shaker for 30 min at 142 rpm. Samples were filtered through Whatman No.

42 filter paper and frozen until analysis on an Agilent Microwave Induced Plasma Spectrometer (Agilent Technologies, Santa Clara, CA, USA).

3.4.7 Soil sampling and analysis for microbial community composition

When field pea, lentil, and chickpea were at approximately 50 to 60 % flowering in the 2018 growing season, additional soil samples were collected for microbial biomass carbon (MB-C) and nitrogen (MB-N) and phospholipid fatty acid analysis (PLFA). Soil for MB-C and MB-N was collected from 0- to 15-cm depth adjacent to the four sampling areas using a Dutch auger. Soil for PLFA analysis was collected at the same time that the nodulation assessment was conducted (section 3.6.1). Plants were carefully excavated and gently shaken to free roots of soil, then a combination of bulk soil adjacent to nodulation assessment plants and root soil was collected for a total of four samples.

Moist samples for MB-C and MB-N were sieved to 2 mm and stored at 4 °C until chloroform fumigation extraction (Voroney et al., 2007) was performed approximately one month after sampling. Soils were covered and incubated at 21 °C for 7 days at approximately 50% water holding capacity. Three 25 g subsamples of incubated soil were placed in an airtight desiccator and fumigated with ethanol-free chloroform for 24 h. Three unfumigated 25 g subsamples were extracted with 80 mL of 0.5 M K_2SO_4 and filtered into vials. After the 24 h incubation was complete, the fumigated subsamples were also extracted with 80 mL of 0.5M K_2SO_4 and filtered into vials. Extracts were frozen until analysis on a Shimadzu TOC-V CPN analyzer (Shimadzu Corp., Kyoto, Japan).

Samples for PLFA analysis were sieved to 2 mm and stored at -80 °C. Soils were freeze-dried and ground using a mortar and pestle. All glassware for the extraction was soaked in 4 % (v/v) Extran 300 (MilliporeSigma, Burlington, MA) soap bath for 2 h, then scrubbed and rinsed

thoroughly with distilled water. The clean glassware was then soaked in 10% hydrochloric acid to remove any remaining lipid debris, then triple-rinsed with distilled water and air-dried. Glassware was baked at 400 °C for 4 h in a muffle furnace. Ultra-high purity N_2 (Praxair Canada Inc., Mississauga, ON) was used for sample evaporation. Standard protocol for PLFA included lipid extraction with a single-phase chloroform mixture, isolation of phospholipids with lipid fractionation using solid phase extraction columns, and methylation of phospholipids to produce fatty acid methyl esters, then analysis using a capillary gas chromatograph (Quideau et al., 2016).

Phospholipid fatty acid extraction was performed according to the modified protocol of White (1979), which was adapted from the original method of Bligh and Dyer (1959) as described in Helgason et al. (2009). Fatty acids were extracted from 4 g of soil using 19 mL of Bligh and Dyer extractant (5 mL chloroform, 10 mL methanol, 4 mL of phosphate buffer consisting of 2.18 g dipotassium phosphate in 250 mL of ultra-pure water). The mixture was centrifuged for 15 min at 1500 rpm. Supernatant was transferred to 50 mL glass vials then 5 mL phosphate buffer and 5 mL chloroform added. The new mixture was vortexed for 30 s. Samples were left overnight at room temperature in the dark. The denser organic phase was transferred into a 15 mL vial and was evaporated under pressure with N_2 at 25 °C. Dried samples were stored at -20 °C until the second step.

Solid phase extraction columns with spigots were conditioned with 5 mL acetone followed by two additions of 5 mL chloroform. Samples were re-dissolved with 1 mL of chloroform after reaching room temperature and transferred into labeled solid phase extraction columns for lipid separation. Neutral and glycolipids were eluted from samples sequentially, with 5 mL chloroform and 5 mL acetone. Phospholipids were eluted with the addition of 5 mL methanol. Eluent was collected into new 15 mL vials. Samples were dried under N_2 and stored at -20 °C.

Lipid methylation was completed by adding 0.5 mL of chloroform and 0.5 mL of methanol to each room temperature sample. After the addition of 1 mL 0.2 M methanolic potassium hydroxide, samples were sealed and placed in a 37 °C water bath for 30 min. Then, 2 mL of hexane, 0.2 mL 5.75% (v/v) acetic acid, and 2 mL of Millipore water (MilliporeSigma, Burlington, MA) were added. Samples were vortexed, then centrifuged at 1500 rpm for 2 min. Ten μL of 0.1 μg μL^{-1} methylated internal standard was added to labeled 4 mL amber vials. The top phase from the centrifuged samples was transferred into the vials. After adding 2 mL of hexane to the lower phase of the sample, it was centrifuged again at 1500 rpm for 2 min. The top phase was transferred again into the amber vials. Samples were evaporated under N_2 and stored at -20 °C.

Fatty acid methyl ester extracts were analyzed using a Bruker Scion 436 gas chromatograph (Scion Instruments, Livingston, UK). Peaks were identified using fatty acid standard and Compass CDS software (Scion Instruments, Livingston, UK). Total microbial biomass was quantified by summing all identified PLFA peaks. Biomarkers based on the PLFA chain length were used to determine the relative abundance of microbial functional groups (Table 3.2).

Table 3.2. Biomarkers used to determine abundance of specific microbial functional groups.

Functional groups	Biomarker(s)	References
Bacteria	i14:0, i15:0, a15:0, i16:0, 16:1ω7c, 10Me16:0, i17:0, a17:0, cy17:0, 10Me17:0, 18:1ω7, 10Me18:0, cy19:0	(Helgason et al., 2010a) (Bååth and Anderson, 2003)
Gram positive	i14:0, i15:0, a15:0, i16:0, i17:0, a17:0	(Helgason et al., 2010b) (Hedrick et al., 2005)
Gram negative	16:1ω7t, 16:1ω9c, 16:1ω7c, 18:1ω7c, 18:1ω9c, cy17:0, cy19:0	(Helgason et al., 2010b) (Macdonald et al., 2004)
Fungi	18:2ω6,9	(Bååth and Anderson, 2003)
Arbuscular mycorrhizal fungi	16:1ω5c	(Olsson, 1999)

3.4.8 Plant sampling for nodulation assessments and ^{15}N analysis

A nodulation assessment (Appendix A.1.) was completed when field pea, lentil and chickpea were between 50 and 60% flowering. Simultaneously, plant health and disease assessments were completed in the field using the plant health scale included in the nodulation assessment criteria (see Appendix, Table A.1). If plants were green and vigorous, they scored a rating of five, and if plants were very chlorotic, they scored a rating of one. A score of zero meant that nodules present were white or green, or no nodules were present on the root system. Disease was determined by visual assessment and gathering field history from the producer. Five to eight plants were randomly selected near each microplot and were carefully excavated to a depth of 30 cm, retaining as much root mass as possible. A total of 20 to 32 plants were collected from each field and were refrigerated at 4 °C until nodulation assessments were completed. In the laboratory, roots of five randomly selected plants were washed and nodules were removed. Nodule colour and

abundance and position on the plant were assessed and final nodulation scores were calculated and reported.

Crops were hand-harvested near physiological maturity. At producer field sites and AAFC Swift Current, 1m^2 microplots were hand-harvested and at AAFC Indian Head, a linear metre strip of mature plants was hand-harvested. Samples of non-N_2 fixing weeds were collected, within a few metres to microplots, as an additional reference source to use in calculating percent N derived from atmosphere (%Ndfa). Using weeds as a reference may provide a more accurate estimate of $\delta^{15}N$ because they utilize the same N pool as the pulse crop and are closer than the reference crop, reducing spatial variability of $\delta^{15}N$. Plant parts were used for natural abundance (NA) ^{15}N measurement, to calculate %Ndfa, yield, nitrogen harvest index (NHI), the total amount of N_2 fixed in seed and straw, and the total amount of N derived from soil (Ndfs).

Plants were air-dried, weighed for dry biomass, and threshed to separate the seeds and straw of field pea, lentil, and chickpea. Plant parts were ground with a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA), then further ground using a ball mill grinder to a fine talc consistency. Reference weeds were ground whole to the same consistency. All samples were encapsulated and analyzed for total N concentration and atom%^{15}N content using a Costech Elemental Combustion system coupled to a Delta V Advantage Mass Spectrometer (Thermo Fisher Scientific Inc, Waltham, MA).

3.4.9 Calculations

Natural ^{15}N abundance of seed and straw samples were calculated using:

$$Rsample = \frac{atom\%^{15}N_{sample}}{100 - atom\%^{15}N_{sample}} \qquad \text{(Eq. 3.1)}$$

$$Rstandard = \frac{0.36637}{100 - 0.36637} \quad \text{(Eq. 3.2)}$$

$$\delta^{15}N\ (‰) = \left[\left(\frac{Rsample}{Rstandard}\right) - 1\right] x\ 1000 \quad \text{(Eq. 3.3)}$$

where the standard is atmospheric N_2 (0.36637 atom% ^{15}N) (Unkovich et al., 2008). Percent Ndfa was calculated using:

$$\%Ndfa = \frac{\delta^{15}N_{\ reference\ plant} - \delta^{15}N_{\ legume}}{\delta^{15}N_{reference\ plant} - B}\ x\ 100 \quad \text{(Eq. 3.4)}$$

where reference plant refers to the non-N_2-fixing plant and the factor B refers to the $\delta^{15}N$ value of an effectively nodulated legume grown in media free of N (Unkovich et al., 1994). For this study, the B values of field pea, lentil, and chickpea were averages from multiple experiments where the values were -0.66, -0.56, and -1.75, respectively (Unkovich et al., 2008).

Amount of N fixed was calculated for seed and straw separately (Hardarson and Danso, 1993) as:

$$Nfixed = \frac{\%Ndfa\ x\ totalN_{fixing}}{100} \quad \text{(Eq. 3.5)}$$

Nitrogen harvest index was calculated by dividing the amount of N in seed by the total amount of N in seed and straw. Total Ndfs was calculated by subtracting N acquired through BNF from total N in the plant (Knight, 2012).

3.4.10 Statistical analysis

Data were analyzed using SAS software (SAS Institute, Inc., version 9.4, Cary, NC). Outliers were identified using box plots and were excluded if any observation was more than two standard deviations from the mean. Data were analyzed as a completely randomized design and were subjected to a two-way analysis of variance (ANOVA) using the PROC GLIMMIX procedure with a significance level of 0.10. The GLIMMIX procedure accounts for normality and

variances in the data sets. The two factors in the ANOVA were site and stubble and were considered fixed. There were two sites in 2017 and seven sites in 2018. Stubble type had two levels, oilseed stubble and cereal stubble. The ANOVA also analyzed the site x stubble interaction. The RANDOM statement with a RESIDUAL effect using site as the error term was used to model residual heterogeneity. Site and stubble were considered the fixed effects. When an effect was significant, the LSMEANS statement was used to facilitate means comparisons. Nodulation assessment data were not statistically analyzed because true field replicates were not collected. Due to the amount of labour required and the high cost per sample, PLFA field replicate samples were combined and only one sample per field was analyzed, therefore no statistical analyses were performed on this data.

3.5 Results

3.5.1 Weather data

Weather data were collected from Environment Canada weather stations nearest the eight locations for the 2017 (Table 3.3) and 2018 (Table 3.4) growing seasons from May to August (Environment Canada, 2011). Total precipitation in 2017 was 18% lower than average at Biggar and Wilkie as compared to the 30-year (1981-2010) historical averages (Table 3.3). Mean daily maximum temperatures in 2017 were similar to historical averages (Table 3.3).

Table 3.3. Weather data for the 2017 field sites during the growing season (May to August) as compared to historical (1981-2010) mean data.

Site	Month	Precipitation	HM[†]	Temperature	HM
		----------------mm--------------		---------------°C---------------	
BG[‡]	May	69.0	44.0	18.7	18.0
	June	34.3	58.6	21.5	22.2
	July	22.4	67.1	25.6	25.1
	August	53.0	48.7	23.7	24.8
	Sum/Mean[§]	**178.7**	**218.4**	**22.4**	**22.5**
WL	May	69.0	36.3	18.7	17.7
	June	34.3	61.8	21.5	21.7
	July	22.4	72.1	25.6	23.7
	August	53.0	45.7	23.7	23.6
	Sum/Mean	**178.7**	**215.9**	**22.4**	**21.7**

[†] HM = historical mean data (1981-2010) was collected from Environment Canada Meteorological Stations at Biggar and Scott, and current weather data for both locations was collected from the nearest station at Scott, SK (Environment Canada, 2011).
[‡] BG = Biggar, WL = Wilkie
[§] Precipitation data is cumulative from May to August; temperature is the mean monthly maximum temperature for the same months.

In 2018, at locations across the province, weather data showed hotter and drier conditions. Generally, the average temperature for May through August was 2.3 °C higher than historical averages across locations except at Biggar, which was only 0.8 °C higher and Davidson which was 2.7 °C higher. The most extreme precipitation differences from the historical averages was at Swift Current, where precipitation was 120 mm less than the 30-year historical average (Table 3.4) Precipitation was evenly distributed throughout the growing season at all locations except at Indian Head, where only 3.9 mm of precipitation accumulated in August (Table 3.4).

Table 3.4. Weather data for the 2018 field sites during the growing season (May to August) and historical (1981-2010) mean data at all field locations.

Soil zone	Site	Nearest station	Month	Precipitation	HM[†]	Temperature	HM
				----------------mm----------------		----------------°C----------------	
Brown	CB[‡]	Elbow	May	29.6	51.2	22.9	17.5
			June	33.6	78.9	24.8	21.8
			July	33.9	53.4	26.2	25.6
			August	32.5	45.2	26.0	25.2
			Sum/Mean	**129.6**	**228.7**	**25.0**	**22.5**
Brown	SC	Swift Current	May	14.9	48.5	21.7	17.5
			June	20.2	72.8	24.0	21.6
			July	32.0	52.6	26.6	25.3
			August	28.0	41.5	26.4	25.2
			Sum/Mean	**95.1**	**215.4**	**24.7**	**22.4**
Dark Brown	BG	Scott	May	29.6	44.0	21.5	18.0
			June	29.6	58.6	23.2	22.2
			July	48.2	67.1	24.6	25.1
			August	23.3	48.7	23.8	24.8
			Sum/Mean[§]	**130.7**	**218.4**	**23.3**	**22.5**
Dark Brown	DV	Elbow	May	29.6	48.3	22.9	17.5
			June	33.6	72.0	24.8	21.8
			July	33.9	64.1	26.2	25.2
			August	32.5	50.4	26.0	24.9
			Sum/Mean	**129.6**	**234.8**	**25.0**	**22.3**

Table 3.4. continued Weather data for the 2018 field sites during the growing season (May to August) and historical (1981-2010) mean data at all field locations.

Soil zone	Site	Nearest station	Month	Precipitation	HM[†]	Temperature	HM
				-----------mm---------		----------°C---------	
Black	IH	Indian Head	May	23.7	51.7	23.0	17.9
			June	90.0	77.4	24.0	22.2
			July	30.4	63.8	25.4	25
			August	3.9	51.2	27.0	24.7
			Sum/Mean	**148.0**	**244.1**	**24.0**	**21.8**
Black	SP	Yorkton	May	14.0	51.3	22.8	17.3
			June	117.3	80.1	23.6	21.7
			July	58.3	78.2	25.0	24.3
			August	31.5	62.2	24.7	23.9
			Sum/Mean	**221.1**	**271.8**	**24.0**	**21.8**
Black	TH	Yorkton	May	14.0	51.3	22.8	17.3
			June	117.3	80.1	23.6	21.7
			July	58.3	78.2	25.0	24.3
			August	31.5	62.2	24.7	23.9
			Sum/Mean	**221.1**	**271.8**	**24.0**	**21.8**

[†] HM = historical mean data (1981-2010) and 2018 data from Environment Canada Meteorological Stations located nearest the sites (Environment Canada, 2011).
[‡] CB = Central Butte, SC = Swift Current, BG = Biggar, DV = Davidson, IH = Indian Head, SP = Springside, and TH = Theodore
[§] Precipitation data is cumulative from May to August; temperature is the mean monthly maximum temperature for the same month.

3.5.2 Soil physical and chemical properties

Soil pH and EC values from the 0- to 15-cm and 15- to 30-cm depths indicated that none of the sites were highly acidic or saline in either year (Table 3.5 and Table 3.6). In 2017, organic C and total N contents were higher at Wilkie than at Biggar (Table 3.5). Levels of nutrient content in each soil were classified as deficient, marginal, optimal, or excessive according to generalized critical limits for cereals and oilseeds (Table A.2.). There were no differences between stubble types at either site for ammonium or nitrate and levels were deficient through optimal. Soil test P was deficient in both stubbles and locations and ranged from 10 to 31 kg ha^{-1} in the 0- to 15-cm depth (Table 3.5). No patterns were observed in either stubble for potassium at Biggar or Wilkie and levels were optimal (Table 3.5). Sulfur was marginal in cereal stubble at Biggar (13 kg ha^{-1} in 0- to 30-cm depth) and was optimal in oilseed stubble (35 kg ha^{-1} in 0- to 30-cm depth). At Wilkie, sulfur was excessive in both oilseed and cereal stubbles with levels of 86 and 1375 kg ha^{-1} in the 0- to 30-cm depth, respectively (Table 3.5). The high sulfur value in the cereal stubble at Wilkie is consistent with high EC values.

In 2018, organic C (OC) and total N (TN) were similar between oilseed and cereal stubbles at the Brown soil zone locations (Table 3.6). At Central Butte, OC contents were 1.32% in the oilseed stubble and 1.35% in the cereal stubble while TN content was 0.14% in both stubbles. At Swift Current, the OC content in oilseed stubbles averaged 1.66% and was 1.68% in the cereal stubbles in the 0- to 15-cm depths (Table 3.6). Total N at Swift Current in all stubbles was 0.17% in the 0- to 15-cm depths. In the Dark Brown soil zone, OC content was higher in both wheat stubbles than in the canola stubbles at Biggar and Davidson (Table 3.6). At Biggar in the 0- to 15-cm depths the wheat 1 stubble had higher OC content than the canola 1 stubble, and the wheat 2 stubble had a higher OC content than the canola 2 stubble (Table 3.6). Total N content in the same

depth in wheat 1 stubble was higher than canola 1, and wheat 2 higher than the canola 2 stubble at Biggar (Table 3.6). At Davidson, OC and TN contents were higher in the wheat stubble than the canola stubble (Table 3.6). No patterns in OC or TN were observed at Indian Head, Springside, or Theodore in the Black soil zone (Table 3.6).

In 2018, there were no obvious differences between stubbles for inorganic N contents across all sites except at Springside, where ammonium content was 0.15 kg ha^{-1} and 0.18 kg ha^{-1} higher and nitrate content was 25 kg ha^{-1} and 54 kg ha^{-1} higher in oat stubble than canola stubble in the 0- to 15- and 15- to 30-cm depths, respectively (Table 3.6). Soil test P content was approximately five times higher in the 0- to 15-cm depth and almost nine times higher in the 15- to 30-cm depth at Swift Current than at Central Butte, averaged across stubbles. At Biggar, no patterns were observed for phosphorus content in either stubble, while at Davidson, phosphorus content in wheat stubble was approximately 18 kg ha^{-1} higher in the 0- to 15-cm depth and 13 kg ha^{-1} higher in the 15- to 30-cm depth than in canola stubble (Table 3.6). Available P content at all locations and stubbles in the Black soil zone indicated deficiency. For potassium content, no patterns or differences between stubbles were observed at 2018 sites (Table 3.6). Sulfur contents were generally marginal to optimal with the exception of the wheat stubble in the 0- to 15-cm depth at Central Butte, which was undetectable (Table 3.6).

Table 3.5. Soil physical and chemical characteristics and plant available macronutrient concentrations sampled from oilseed and cereal stubble prior to seeding field pea at two locations in the Dark Brown soil zone in 2017.

Soil Zone	Site	Stubble	Depth	Texture	pH	EC	OC†	TN	NH_4^+	NO_3^-	P	K	S
						mS cm^{-1}	------ % -----		-----------------kg ha^{-1}---------------				
Dark Brown	BG	Canola	0-15	Sandy loam	6.1	0.16	1.78	0.18	0.5	15	19	725	17
			15-30		6.2	0.16	1.59	0.16	0.6	22	12	622	18
		Barley	0-15	Sandy loam	6.2	0.11	1.46	0.14	0.3	11	31	518	7
			15-30		6.6	0.14	1.10	0.11	0.4	11	11	353	6
Dark Brown	WL	Canola	0-15	Loam	6.4	0.24	3.09	0.29	0.7	18	13	434	29
			15-30		7.0	0.44	2.45	0.22	0.6	25	5	323	57
		Wheat	0-15	Silt loam	6.3	0.93	3.77	0.35	0.7	29	10	690	553
			15-30		6.7	1.26	2.76	0.27	0.8	29	15	499	822

† OC = organic C, TN = total N, BG = Biggar, WL = Wilkie

Table 3.6. Soil physical and chemical characteristics and plant available macronutrient concentrations sampled from oilseed and cereal stubbles prior to seeding pulse crops at seven locations in three soil zones of Saskatchewan in 2018.

Soil Zone	Site	Stubble	Depth	Texture	pH	EC	OC†	TN	NH_4^+	NO_3^-	P	K	S
			cm			mS cm^{-1}	------ % -----		------------------kg ha^{-1}---------------				
Brown	CB	Canola	0-15	Sandy loam	7.4	0.54	1.32	0.14	0.42	24	16	932	198
			15-30		7.7	0.88	0.94	0.11	0.56	21	6	537	376
		Wheat	0-15	Sandy loam	7.3	0.19	1.35	0.14	0.39	21	12	642	0
			15-30		8.0	0.26	1.02	0.10	0.43	17	3	428	15
Brown	SC	Mustard 1	0-15	Silt loam	7.3	0.16	1.64	0.17	0.51	31	57	400	6
			15-30		7.3	0.20	1.62	0.16	0.51	37	41	505	8
		Wheat 1	0-15	Silt loam	6.0	0.10	1.76	0.17	0.49	31	70	418	5
			15-30		6.1	0.12	1.56	0.16	0.48	37	34	374	5
		Mustard 2	0-15	Silt loam	6.1	0.11	1.67	0.17	0.50	28	79	400	7
			15-30		6.2	0.12	1.53	0.15	0.40	36	38	361	9
		Wheat 2	0-15	Loam	6.3	0.11	1.59	0.16	0.49	29	69	459	7
			15-30		6.4	0.14	1.34	0.14	0.51	32	41	397	4
Dark Brown	BG	Canola 1	0-15	Silt loam	7.7	0.28	0.97	0.13	0.46	24	10	421	15
			15-30		8.0	0.22	1.47	0.09	0.33	19	4	286	9
		Wheat 1	0-15	Silt loam	7.6	0.36	1.39	0.21	0.52	44	74	665	15
			15-30		7.9	0.29	1.30	0.12	0.46	41	31	558	13
		Canola 2	0-15	Sandy loam	7.6	0.48	2.59	0.24	0.53	48	28	700	27
			15-30		7.9	0.37	1.61	0.16	0.51	35	9	562	378
		Wheat 2	0-15	Silt loam	7.8	0.21	3.24	0.30	0.63	44	21	688	24
			15-30		7.7	0.37	2.29	0.21	0.53	36	13	447	23
Dark Brown	DV	Canola	0-15	Loam	7.5	0.31	1.67	0.18	0.63	52	10	499	23
			15-30		7.6	0.30	1.54	0.14	0.71	65	5	459	17
		Wheat	0-15	Silt loam	7.7	0.27	2.52	0.23	0.85	49	28	641	15
			15-30		7.7	0.29	2.04	0.20	0.67	52	18	504	19

Table 3.6. continued Soil physical and chemical characteristics and plant available macronutrient concentrations sampled from oilseed and cereal stubbles prior to seeding pulse crops at seven locations in three soil zones of Saskatchewan in 2018.

Soil Zone	Site	Stubble	Depth	Texture	pH	EC	OC	TN	NH_4^+	NO_3^-	P	K	S
						mS cm^{-1}	------ %	-----	--------------------	kg ha^{-1}			---------------
Black	IH	Canola	0-15	Sandy loam	7.6	0.35	2.43	0.22	1.01	37	15	505	47
			15-30		7.6	0.36	1.35	0.14	0.93	30	4	332	36
		Wheat	0-15	Sandy loam	8.0	0.31	2.65	0.21	0.79	37	14	515	20
			15-30		8.0	0.40	1.68	0.14	0.67	33	4	290	74
Black	SP	Canola	0-15	Sand	8.2	0.25	1.40	0.09	0.30	22	2	132	17
			15-30		8.2	0.23	1.07	0.10	0.23	14	3	118	13
		Oat	0-15	Sandy loam	7.5	0.48	2.90	0.09	0.48	47	4	268	111
			15-30		7.7	0.46	2.21	0.16	0.38	68	2	318	138
Black	TH	Canola	0-15	Sandy loam	7.7	1.16	5.24	0.44	0.88	32	8	1504	123
			15-30		7.6	1.50	3.77	0.30	0.62	23	3	345	253
		Oat	0-15	Sandy loam	7.9	0.47	4.23	0.34	0.60	32	15	233	88
			15-30		7.9	0.45	3.61	0.27	0.55	28	6	282	88

† CB = Central Butte, SC = Swift Current, BG = Biggar, DV = Davidson, IH = Indian Head, SP = Springside, and TH = Theodore

3.5.3 Soil biological properties

Total microbial abundance measured by PLFA analysis was highest in the Black soil zone with an average of 106.9 nmol g^{-1} soil, followed by the Dark Brown soil zone with an average of 79.5 nmol g^{-1} soil, and was lowest in the Brown soil zone averaging 50.0 nmol g^{-1} soil (Table 3.7). No observable differences between microbial functional groups were found at Central Butte or Swift Current (Table 3.7). In the Dark Brown soil zone all levels of functional group biomarkers and total microbial abundance were highest in the wheat-field pea rotation sequence at Biggar, however at Davidson, all functional group biomarkers except AMF were higher in the canola-lentil rotation sequence than in the wheat-lentil rotation sequence (Table 3.7). In the Black soil zone, there were no observable differences in functional group biomarker levels or total microbial abundance at Indian Head (Table 3.7). At Springside, AMF content (6 nmol g^{-1} soil) was highest out of all locations (Table 3.7). At Theodore, gram positive, gram negative, and actinobacteria biomarkers were higher in the canola-field pea rotation sequence (165 nmol g^{-1} soil) and out of all locations, had the highest total microbial abundance (Table 3.7).

An interaction between site and stubble affected MB-C (p=0.0062) and MB-N (p=0.0452) (Table 3.8). No significant differences between stubbles at each location occurred except at Theodore, where MB-C in the oilseed stubble was 235 µg g^{-1} soil higher than in cereal stubble. No significant differences for MB-N occurred between any stubbles at any site location. Generally, MB-C and MB-N were greater in oilseed stubbles than in cereal stubbles except at Central Butte, where MB-C in cereal stubble was higher, and at Biggar, where MB-C and MB-N were both greater in cereal stubble than in oilseed stubble (Table 3.8).

Table 3.7. Phospholipid fatty acid analysis (PLFA) biomarker content (nmol g^{-1} soil) sampled from pulse crops grown on oilseed or cereal stubble. Bulk soil surrounding roots (0- to 15-cm depth) was sampled when plants were at approximately 50% flowering in 2018.

Soil Zone	Site	Rotation Sequence	$G^{+\dagger}$	G^{-}	ACT	AMF	FUN	Total PLFA	F:B
			-----nmol g^{-1} soil-----						
Brown	CB	Cn-FP	12	14	6	2	1	53	0.08
		W-FP	10	13	6	2	1	48	0.07
Brown	SC	M-CP	10	15	6	2	1	49	0.05
		W-CP	14	17	6	1	1	60	0.05
		M-L	11	12	5	1	1	46	0.05
		W-L	11	13	6	1	1	47	0.04
Dark Brown	BG	Cn-FP	13	21	7	3	3	68	0.16
		W-FP	22	29	11	4	2	96	0.07
		Cn-L	20	24	7	3	2	88	0.09
		W-L	19	24	10	3	2	86	0.07
Dark Brown	DV	Cn-L	17	26	10	3	4	87	0.14
		W-L	11	16	5	3	1	52	0.09
Black	IH	Cn-FP	16	20	7	3	2	70	0.07
		W-FP	16	22	8	3	2	72	0.07
Black	SP	Cn-FP	25	40	11	6	2	121	0.06
		O-FP	27	35	11	5	3	121	0.08
Black	TH	Cn-FP	36	52	18	1	3	165	0.05
		O-FP	19	31	8	4	2	92	0.09

† G^{+} = gram positive bacteria, G^{-} = gram negative bacteria, ACT = actinobacteria, AMF = arbuscular mycorrhizal fungi, FUN = fungi, F:B = fungi to bacteria ratio, CB = Central Butte, SC = Swift Current, BG = Biggar, DV = Davidson, IH = Indian Head, SP = Springside, and TH = Theodore, FP = field pea, CP = chickpea, L = lentil, Cn = canola, M = mustard, O = oat, W = wheat

Table 3.8. Microbial biomass C (MB-C) and N (MB-N) in soil sampled from a pulse crop grown on oilseed (canola or mustard) or cereal (wheat or oat) stubbles measured by chloroform fumigation extraction. Soils were sampled in 2018 at mid- to late-flowering of the pulse crop.

Soil zone	Site	Stubble	MB-C	MB-N
	Main effects		mg C kg^{-1} soil	mg N kg^{-1} soil
Brown	CB[†]		370bc[‡]	25de
Brown	SC		154d	17e
Dark Brown	BG		432b	40bc
Dark Brown	DV		631a	68a
Black	SP		291c	35cd
Black	TH		425b	57ab
		OIL	415a	47a
		CER	352b	34b
	Interactions			
Brown	CB	OIL	356bc	29c
		CER	384bc	22c
Brown	SC	OIL	180d	19c
		CER	127d	15c
Dark Brown	BG	OIL	394bc	37c
		CER	470bc	42bc
Dark Brown	DV	OIL	724a	88a
		CER	539abc	48abc
Black	SP	OIL	297cd	39bc
		CER	285cd	32c
Black	TH	OIL	542ab	67ab
		CER	307bcd	46abc
		Probability		
	Site		<0.0001	<0.0001
	Stubble		0.0080	0.0008
	Site*Stubble		0.0062	0.0452

[†]CB= Central Butte, SC = Swift Current, BG = Biggar, DV = Davidson, SP = Springside, and TH = Theodore

[‡]Values are means (n=4). Means followed by the same letter are not significantly different ($p \geq 0.05$)

3.5.4 Nodulation assessments

Effective nodulation was observed in both rotation sequences at Biggar in 2017 as indicated by high total nodulation assessment scores (scores between 11 and 13); however, at Wilkie, nodulation was rated less effective (scores between 7 and 10) in the wheat-field pea rotation sequence and poor nodulation was observed in the canola-field pea rotation sequence (scores between 1 and 6) (Table 3.9). Upon entering the canola-field pea field at Wilkie, plants looked

stunted and yellowish-green. When plants were excavated, discoloured roots indicated a root rot disease was present, and an infestation of *Aphanomyces euteiches* was suspected. Soil was sent for testing and the outcome was positive for the disease.

Less effective nodulation scores due to colour and abundance of nodules, and nodule position were observed at Swift Current in the mustard-chickpea and mustard-lentil sequences (Table 3.9). Effective nodulation scores were observed at Biggar and Davidson in all rotation sequences where the crop was healthy, had actively fixing nodules in good number, on either the root crown or on lateral roots of the plant (Table 3.9). Poor nodulation scores were assigned to the plants from the canola-field pea rotation sequence at Indian Head, where all scoring categories were low. Plants were green and relatively small and did not show any symptoms of disease. Nodules were few in number and were white or green in colour, and nodules were located on lateral roots only (Table 3.9). Less effective nodulation was also found at Indian Head in the wheat-field pea sequence and at Springside in both rotation sequences (Table 3.9). These slightly lower scores were not due to poor plant growth or presence of disease. The colour (leghemoglobin activity) and abundance of nodules and nodule position scores were low.

Table 3.9. Nodulation assessment scores of nodules for field pea, lentil, or chickpea grown on oilseed or cereal stubbles, sampled at approximately 50% flowering at 2017 and 2018 field sites.

Soil Zone	Site	Rotation Sequence	Assessment criteria of nodules			
			PG[†]	CA	NP	Total
2017						
Dark Brown	BG	Cn-FP[‡]	5.00	5.00	2.50	12.50
		B-FP	5.00	5.00	2.25	12.25
Dark Brown	WL	Cn-FP	3.00[¶]	0.25	0.75	4.00
		W-FP	5.00	1.50	1.50	8.00
2018						
Brown	CB	Cn-FP	5.00	5.00	3.00	13.00
		W-FP	5.00	5.00	3.00	13.00
Brown	SC	M-CP	5.00	3.00	2.25	10.25
		W-CP	5.00	4.00	2.25	11.25
		M-L	5.00	3.50	1.00	9.50
		W-L	5.00	5.00	1.50	11.50
Dark Brown	BG	Cn-FP	5.00	5.00	3.00	13.00
		W-FP	5.00	5.00	3.00	13.00
		Cn-L	5.00	3.00	3.00	11.00
		W-L	5.00	5.00	3.00	13.00
Dark Brown	DV	Cn-L	5.00	5.00	3.00	13.00
		W-L	5.00	5.00	3.00	13.00
Black	IH	Cn-FP	3.50	1.25	0.75	5.50
		W-FP	5.00	1.50	1.00	7.50
Black	SP	Cn-FP	5.00	1.00	1.00	7.00
		O-FP	5.00	1.00	1.00	7.00
Black	TH	Cn-FP	5.00	5.00	3.00	13.00
		O-FP	5.00	5.00	1.00	11.00

[†] PG = Plant growth and vigour, CA = Colour and abundance, NP = nodule position, CB= Central Butte, SC = Swift Current, BG = Biggar, DV = Davidson, WL = Wilkie, IH = Indian Head, SP = Springside, and TH = Theodore, FP = field pea, CP = chickpea, L = lentil, Cn = canola, M = mustard, B = barley, O = oat, W = wheat,

[‡]Field pea on canola stubble at Wilkie in 2017 had a severe *Aphanomyces euteiches* infestation which affected root growth of the crop.

3.5.5 Estimates of nitrogen derived from atmosphere and nitrogen acquisition

In 2017, an interaction between site and stubble affected %Ndfa in seed (p=0.0323) (Table 3.10). There were no significant differences between stubbles at Biggar and Wilkie, but between locations, %Ndfa in field pea seed grown on oilseed stubble was higher at Biggar than at Wilkie (Table 3.10). Percent Ndfa in straw was affected by site only (p<0.0001); %Ndfa was lower at

Wilkie (46%) than at Biggar (77%) (Table 3.10). Nitrogen harvest index was affected by an interaction between site and stubble (p=0.0122) where the cereal rotation sequence at Wilkie had the lowest NHI (0.77) (Table 3.10). Aboveground N was significantly higher at Wilkie in field pea grown on cereal stubble (p=0.0007) (Table 3.10). Nitrogen fixed was not different between field pea grown on oilseed or cereal stubbles at Biggar, but at Wilkie, was higher in cereal stubble (93 kg ha^{-1}) than in oilseed stubble (27 kg ha^{-1}) (p<0.0001). Nitrogen derived from soil was affected by an interaction between site and stubble but no differences between stubbles were found (p=0.0407) (Table 3.10). Field pea grown on cereal stubble at Wilkie derived more N from soil, which corresponds to the high ABG-N, but not the high amount of fixed N (Table 3.10). Seed and straw yields were not affected by site, stubble or an interaction between the two factors; however, the higher seed and straw yields in field pea grown on cereal stubble at Wilkie could account for the higher ABG-N and partitioning of N (Table 3.10).

In 2018, an interaction between site and stubble affected %Ndfa in seed (p= 0.0006) and straw (p= 0.0175) (Table 3.11). Percent Ndfa in seed and straw was higher in cereal stubble at all locations, except Davidson and Theodore, where %Ndfa was higher in oilseed stubble (Table 3.11). Between locations there was no difference between stubbles, except for %Ndfa in seed at Central Butte, which was higher than at Swift Current (Table 3.11). Nitrogen harvest index was affected by an interaction between site and stubble; however, the values were generally similar.

Aboveground N was affected by site (p<0.0001) and by stubble (p=0.0396) independently (Table 3.11). Aboveground N was higher in cereal stubble (126 kg ha^{-1}) than in oilseed stubble (115 kg ha^{-1}) (Table 3.11). The amount of N fixed was affected by site (p<0.0001) (Table 3.11). Nitrogen derived from soil was affected by an interaction between site and stubble (p=0.0302)

(Table 3.11). Seed yield was affected by site independently ($p<0.0001$) and straw yield was affected by an interaction between site and stubble ($p=0.0172$) (Table 3.11).

In the Brown soil zone, ABG-N was almost twice as high at Central Butte (127 kg ha^{-1}) than at Swift Current (62 kg ha^{-1}) and fixed N at Central Butte (102 kg ha^{-1}) was three-fold higher than the amount at Swift Current (30 kg ha^{-1}). Nitrogen derived from soil was not different between stubbles or locations at Central Butte and Swift Current (Table 3.11). Seed yield was higher at Central Butte (3206 kg ha^{-1}) than at Swift Current (1508 kg ha^{-1}) (Table 3.11). Straw yields were higher in field pea grown on cereal stubble at Central Butte (3483 kg ha^{-1}) and yields in both stubble at Swift Current (1383 kg ha^{-1} on oilseed stubble and 1496 kg ha^{-1} on cereal stubble) (Table 3.11). In the Dark Brown soil zone, ABG-N was similar between Biggar and Davidson (Table 3.11). Fixed N was higher at Biggar (106 kg ha^{-1}) than at Davidson (79 kg ha^{-1}), and Ndfs was higher in pulse crops grown on cereal stubble at Davidson (93 kg ha^{-1}) than at Biggar (37 kg ha^{-1}), which could account for the higher ABG-N occurring at Davidson (164 kg ha^{-1}) than at Biggar (149 kg ha^{-1}). No significant differences between seed yield and straw yield were found between stubbles at Biggar and Davidson. In the Black soil zone, ABG-N at Springside (160 kg ha^{-1}) was higher than at Theodore (114 kg ha^{-1}) and was twice the amount found at Indian Head (69 kg ha^{-1}) (Table 3.11). Fixed N at each location followed a similar trend to ABG-N where Springside had the highest amount (103 kg ha^{-1}), followed by Theodore (67 kg ha^{-1}) and the lowest at Indian Head (45 kg ha^{-1}). Nitrogen derived from soil was not different between stubbles at Springside, Theodore, or Indian Head (Table 3.11). Seed yield at Springside was 4021 kg ha^{-1}, at Theodore was 2565 kg ha^{-1} and at Indian Head was 1556 kg ha^{-1} which is consistent with the trend of ABG-N and fixed N. Straw yields were similar between stubbles at Springside and Theodore but were different between Springside and Indian Head (Table 3.11).

Table 3.10. Nitrogen acquisition and yields of field pea grown on oilseed (canola) or cereal (wheat or barley) stubbles at two sites in the Dark Brown soil zone in 2017.

Site	Stubble	%Ndfa† seed	%Ndfa straw	NHI	ABG-N	Fixed N	Ndfs	Seed yield	Straw yield
Main effects		-----------------%-----------------			-------------------------------kg ha^{-1}-------------------------------				
BG		59a‡	77a	0.80a	105b	66a	38b	2200a	2869a
WL		38b	46b	0.79a	147a	60a	86a	3297a	3529a
	OIL	47a	57a	0.80a	99b	49b	50b	2197a	2629a
	CER	50a	65a	0.78b	152a	77a	75a	3300a	3768a
Interactions									
BG	OIL	62a	77a	0.80a	108b	71b	38b	2254a	2968a
	CER	56ab	76a	0.79a	101b	62b	39b	2147a	2769a
WL	OIL	32c§	37b	0.81a	90b	27c	63ab	2140a	2290a
	CER	44bc	55b	0.77b	203a	93a	110a	4455a	4768a
---------------*Probability*---------------									
Site		**<0.0001**	**<0.0001**	0.1092	**0.0078**	0.2368	**0.0006**	0.1592	0.2755
Stubble		0.4251	0.1297	**0.0004**	**0.0019**	**<0.0001**	**0.0322**	0.1583	0.1666
Site*Stubble		**0.0323**	0.0765	**0.0122**	**0.0007**	**<0.0001**	**0.0407**	0.1447	0.1426

†%Ndfa = percent nitrogen derived from atmosphere, NHI = nitrogen harvest index, ABG-N = aboveground nitrogen, Ndfs = N derived from soil, BG = Biggar, WL = Wilkie, OIL = oilseed stubble, CER = cereal stubble

‡Values are means (n=4). Means followed by the same letter are not significantly different (P>0.05).

§A severe *Aphanomyces euteiches* infestation was reported at Wilkie and affected root growth and nodulation of the crop, therefore affecting %Ndfa in seed and straw and subsequent calculations of NHI, Total N, Fixed N, and Ndfs.

Table 3.11. Nitrogen acquisition and yields of pulse crops (field pea, lentil or chickpea) grown on oilseed (canola or mustard) or cereal (wheat or oat) stubbles at seven sites in three soil zones in 2018.

Soil zone	Site	Stubble	%Ndfa seed	%Ndfa straw	NHI	ABG-N	Fixed N	Ndfs	Seed yield	Straw yield
	Main effects		----------------%--------------			----------------------------kg ha^{-1}-------------------------				
Brown	CB		79a[‡]	90a	0.84a	127b	102a	25c	3206b	3134bc
Brown	SC		47b	60b	0.83a	62c	30c	32c	1508c	1439d
Dark Brown	BG		66ab	86a	0.83a	149ab	106a	45bc	3250b	3565ab
Dark Brown	DV		46b	59b	0.79b	164a	79ab	85a	3388ab	3261abc
Black	IH		66ab	71ab	0.81ab	69c	45bc	24c	1556c	1847cd
Black	SP		61b	90a	0.83a	160a	103a	57ab	4021a	3692a
Black	TH		52b	82a	0.81ab	114b	67ab	47bc	2565b	2864bc
		OIL	60a	74a	0.82a	115b	72a	43a	2713a	2709a
		CER	60a	80a	0.82a	126a	80a	46a	2856a	2948a
	Interactions									
Brown	CB	OIL	78a	89a	0.83a	115ab	91ab	24b	2983abc	2785bc
		CER	80a	92a	0.84a	138a	113ab	25b	3430ab	3483ab
Brown	SC	OIL	44b	48c	0.83a	61b	26d	35b	1518d	1383c
		CER	50b	72abc	0.82a	63b	35cd	28b	1498d	1496c
Dark Brown	BG	OIL	59ab	83ab	0.83a	138a	89ab	53ab	3146abc	3151abc
		CER	74ab	88a	0.83a	160a	123a	37b	3354abc	3979a
Dark Brown	DV	OIL	51ab	67abc	0.81ab	164a	88abc	76ab	3405abc	3290abc
		CER	40b	51bc	0.78b	164a	70abcd	93a	3370abc	3233abc

Table 3.11. continued Nitrogen acquisition and yields of pulse crops (field pea, lentil or chickpea) grown on oilseed (canola or mustard) or cereal (wheat or oat) stubbles at seven sites in three soil zones in 2018.

Soil zone	Site	Stubble	%Ndfa seed	%Ndfa straw	NHI	ABG-N	Fixed N	Ndfs	Seed yield	Straw yield
			----------------%--------------			----------------------------kg ha^{-1}-------------------------				
Black	IH	OIL	57ab	66abc	0.79ab	60b	34cd	26b	1332d	1675c
		CER	75ab	76abc	0.82ab	78b	57bcd	21b	1780cd	2019c
Black	SP	OIL	56ab	82ab	0.83a	162a	98ab	64ab	3970ab	3935a
		CER	65ab	99a	0.83a	158a	108ab	49b	4073a	3450ab
Black	TH	OIL	71ab	85a	0.82ab	104ab	78abcd	26b	2640abcd	2745bc
		CER	34b	79ab	0.79ab	124ab	56bcd	68ab	2490bcd	2983abc
Probability										
Site			**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Stubble			0.9555	0.1151	0.4443	**0.0396**	0.1325	0.5526	0.3479	0.1555
Site*Stubble			**0.0006**	**0.0175**	**0.0434**	0.5386	0.2496	**0.0302**	0.6304	**0.0172**

†%Ndfa = percent nitrogen derived from atmosphere, NHI = nitrogen harvest index, ABG-N = aboveground nitrogen, Ndfs = N derived from soil, CB = Central Butte, SC = Swift Current, BG = Biggar, DV = Davidson, IH = Indian Head, SP = Springside, and TH = Theodore, OIL = oilseed stubble, CER = cereal stubble

‡Values are means (n=4). Means followed by the same letter are not significantly different (p >0.05).

3.6 Discussion

3.6.1 Factors affecting biological nitrogen fixation

Multiple factors that affect crop growth and in turn, BNF exist and by characterizing each site location, such factors can be identified. Soil properties that are important for crop growth, rhizobial growth and survival, nodulation, and BNF were addressed. True field replicates were not collected for certain soil properties, and if replicates were created in the lab, pseudo-replication would have been introduced. Although statistical analyses were not performed, the soil properties characterized can be subjectively analyzed in relation to BNF.

Growing season conditions were near normal in 2017 but in 2018 were hotter and drier than historical averages. Precipitation during May, June and July is particularly important for crop yield (Campbell et al., 1988) and the warmer than average temperatures and below average precipitation may have created drought conditions, which could contribute to lower yields. There is a strong linear relationship that exists between water use and crop yield for chickpea, field pea, and lentil (Miller et al., 2002; Angadi et al., 2008). Pulse crop growth may be greatly depressed both by intermittent drought, which could occur at any time during the growing season if rainfall is inadequate, or by terminal drought, which occurs when soil moisture is depleted enough to cause crop senescence (Saxena et al., 1993; Wery et al., 1994). Any factor which slows or disrupts phloem flow within the plant can potentially have a large influence on nodule physiology. The rate of phloem flow to nodules may be decreased early on during development of a water deficit, therefore decreasing carbon input and water flow to a nodule (Serraj et al., 1999). At Indian Head, only 3.9 mm of precipitation occurred in August compared to the historical mean of 51.2 mm. Nodulation number and BNF rates generally peak during early-to mid-flowering stage (Voisin et al., 2003) and BNF slows with the onset of pod filling (Salon et al., 2001). Yields at Indian Head

reflect the low August precipitation, but it is likely that BNF was not affected as %Ndfa at Indian Head is comparable to other locations. By August, the crop at Indian Head had finished flowering, which is when peak nodulation would occur, and was entering pod-filling. At Swift Current, precipitation throughout the growing season was lower than the historical average and yields and BNF were among the lowest reported values. In another study at Swift Current, BNF and yields were higher in a wetter year than in a drier year (Hossain et al., 2016), similar to the low yields and BNF observed at Swift Current in the current study.

Pre-seeding soil fertility across locations was variable. Pulse crops may respond to small amounts of starter fertilizer N applied at the time of seeding, which may alleviate early N deficiencies experienced by the plant after seed N has been fully utilized but before BNF occurs (Sprent and Minchin, 1983). In the current study all soils, with the exception of the barley stubble at Biggar in 2017, had over 37 kg N ha^{-1} in the 0- to 30-cm depths rendering a starter N application unnecessary. In a study involving fertilizer application to field pea, rates less than 40 kg N ha^{-1} had no significant effects on nodulation or BNF and higher rates of applied N replaced fixed N which lead to the conclusion that starter N was not necessary (Clayton et al., 2004). Optimal and even excessive levels of inorganic N across locations according to the generalized critical limits (Table A.2.) may have either contributed more to yield or may have hindered BNF.

It is important to note that the generalized critical limits of macronutrients used for broad comparison are for cereal and oilseed crops, not pulse crops. In two out of three study years, Hossain et al. (2016) found a significant negative correlation between BNF of chickpea, lentil, and pea, soil N uptake, and soil mineral N. In the same study, soil N in spring measured 42 kg ha^{-1} in one year and 9 kg ha^{-1} in the next, with additional starter fertilizer N applied at a rate of 9.5 kg N ha^{-1} (Hossain et al., 2016). It is possible that reduced BNF occurred at locations in the current study

because of optimal to excessive levels of inorganic N. Furthermore, inorganic N measurements taken before seeding do not account for N released through mineralization of OM throughout the growing season (Knight et al., 2010). In 2018, phosphorus content across locations was deficient except at Swift Current in all stubbles, and the wheat 1 stubble at Biggar, both had optimal levels. There were no observable differences between stubbles at each site location that had deficient levels. In a laboratory study using P-deficient soil from the Brown soil zone, yellow pea emergence was not hindered by seed-row placed P when rates were below 20 kg P_2O_5 ha^{-1}. The P was added in the form of monoammonium phosphate (Qian and Schoenau, 2010).

Although seed-row placed or side-banded P fertilizer have been recommended for small grains production on the prairies (Qian and Schoenau, 2010), chickpea and lentil yields were enhanced but BNF was not affected (Bremer et al., 1988; Walley et al., 2005). It is important to note that all field management and nutrient input decisions at sites were under producers' or AAFC control. Potassium levels were optimal at all locations except Springside, where levels were deficient in oilseed stubble and marginal in cereal stubble and at Theodore, where levels were excessive in oilseed stubble and marginal in cereal stubble.

Sulfur levels across locations were variable and ranged from severely deficient to marginal to adequate. Pulse crop growth may be affected by S through its effect on BNF by *Rhizobium* bacteria (Scherer and Lange, 1996). Under S deficiency, BNF may be affected for several reasons: root nodule number and size is reduced, nitrogenase activity is lower, ATP and glucose supply to nodules may be reduced, and ferredoxin supply to nodules is hindered (Scherer, 2008). In a study using sites across Germany, S fertilization did not appear necessary when pea crops yielded up to 4100 kg ha^{-1} as crops appeared to use existing soil S (Pötzsch et al., 2019); however, S fertilization for legumes should be recommended if S delivery from soil OM is not sufficient to cover S demand

(Scherer, 2008). In a study examining the effects of S fertilization on both yield and BNF, seed yield of pea was increased markedly by the addition of S up to an optimal level of 25 mg S kg soil^{-1} (Zhao et al., 1999). In the same study, nodules were visibly fewer and smaller in S-deficient plants and the concentration of S in roots of S-sufficient pea plants was 2.6 to 4.4-fold higher than in shoots, suggesting the high requirement of S for the functions of nodules (Zhao et al., 1999).

Microbial biomass is defined as the part of the OM in soil that constitutes living microorganisms (Joergensen, 1995) and when measured can be used to estimate the size of a pool for the delivery of nutrients (Smith and Paul, 1990). Microbial biomass can also provide an indirect indication of how the size of a microbial community is affected by agronomic practices, i.e. rotation sequence. Microbial biomass C and N rates appeared to be affected more by site than by stubble even though there was an interaction between site and stubble. Microbial biomass C was higher in soil from pulse crops grown on canola stubble than cereal stubble by 235 mg C kg^{-1} soil at Theodore, 185 mg C kg^{-1} soil at Davidson, and 14 mg C kg^{-1} soil at Springside. The quantity and quality of the labile C pool is a key driver of soil microbial community activity and community structure (Breulmann et al., 2012). The higher organic C content at Theodore, Davidson, and Springside may partially explain MB-C levels. At all other sites MB-C was higher in soil from pulse crops grown on cereal stubble.

Microbial biomass N was only slightly higher levels in soil from pulse crops grown on oilseed stubbles than cereal stubbles at all locations except Biggar. A similar trend was seen in total PLFA biomarker levels at Theodore and Davidson, where biomarkers in soil from pulse crops grown on oilseed stubble were 73 nmol g^{-1} soil and 35 nmol g^{-1} soil higher than soil from pulse crops grown on cereal stubble at each location, respectively. Total PLFA biomarkers did not follow the same pattern at Springside and there was no difference in total PLFA biomarkers in either stubble. The

availability of C and water strongly governs the activities of specific microbial populations and functions (Bossio and Scow, 1995). However, since organic C levels in oilseed stubble were higher at Theodore, but not at Springside or Davidson, and precipitation was greater at Springside and Theodore, these circumstances may only partially explain the conditions for MB-C to be higher in oilseed stubble at these locations. Previous crop residue will affect soil microbial composition as the rate of decay and the amount of nutrient released to soil depends on crop species (Gan et al., 2011b); in this study's case, the amount of nutrient released by oilseed or cereal stubble. Rhizodeposition by pulse crop roots also may be partially responsible for differences in soil microbial community as soil microbes utilize rhizo-deposits as a C source (Bais et al., 2006).

There was also a large difference between MB-C at Central Butte and Swift Current where overall MB-C was two-fold higher at Central Butte than at Swift Current. This may be due to differences in applications of fungicides where only one application was made at Central Butte (J. Schoenau, personal communication, 2018) and four applications were made at Swift Current (L. Poppy, personal communication, 2020). Soils with lower clay and organic C content such as at Central Butte and Swift Current have a lower adsorption capacity for pesticides and a higher potential for bioavailability to soil microbes (Ahtiainen et al., 2003). Furthermore, high disease pressure such as ascochyta blight, can lead to abundant fungicide use (Gan et al., 2006). Pesticide use may adversely affect agriculturally important microorganisms such as N_2-fixing bacteria, and reduce the performance of agroecosystems overall (Gaind et al., 2007). A study at Swift Current on the effects of foliar fungicide on non-target organisms found that disease control treatments negatively impacted chickpea nodule size but not function, whether or not the host plant was affected by ascochyta blight (Yang et al., 2012).

3.6.2 Biological nitrogen fixation and nitrogen acquisition

In 2017, %Ndfa of field pea grown on oilseed stubble was higher than cereal stubble at Biggar but not at Wilkie. Aboveground N and the amount of N fixed were also higher in pulse crops grown on oilseed stubble at Biggar. Aboveground N, amount of N fixed, and Ndfs were higher at Wilkie in field pea grown on cereal stubble. These higher amounts reflect almost double the seed and straw yields more so than BNF and Wilkie appears to be more reliant on soil N in cereal stubble. It is important to note that due to an *Aphanomyces euteiches* infestation, the field pea grown on oilseed stubble at Wilkie was chlorotic aboveground and belowground, lacked a healthy root structure that affected plant growth. *Aphanomyces euteiches* is a water mould that was first detected in Saskatchewan in 2012 and causes chlorosis of the plant and poor root development in field pea and other pulse crops (Banniza et al., 2013). *Aphanomyces euteiches* can cause severe root damage to the host crop at any time during its growth (Wu et al., 2018). The difference between BNF of field pea grown on oilseed and cereal stubbles may have been less had the crop at Wilkie not been infected by this disease.

Biological nitrogen fixation (%Ndfa) of pulse crops was lower when grown on oilseed stubble compared to cereal stubble at all locations except Davidson and Theodore in 2018. Differences in microbial populations may be responsible for the differences in %Ndfa at these two locations (Knight, 2012). The patterns that persist in MB-C and total PLFA biomarkers may explain the differences in BNF at Davidson and Theodore. Differences in BNF in seed and straw were most pronounced between Central Butte and Swift Current, where %Ndfa of pulse crop seed and straw was higher in both stubbles at Central Butte. The pesticide applications discussed previously may have impeded nodulation and therefore affected BNF at Swift Current. The results from the current study are congruent with a controlled environment study using soil from Swift Current where canola grown immediately before chickpea, lentil, or field pea resulted in lower

amounts of N fixed compared to wheat (Knight, 2015). In a companion field experiment, lentil and chickpea in rotation with mustard had lower BNF, less biologically fixed N contributing to seed N, and were among the lowest producing rotations (Knight, 2015).

Global average %Ndfa values for pea, chickpea, and lentil are 65 %, 58 %, and 65 %, respectively (Peoples et al., 2009). In a meta-analysis of studies conducted in the Northern Great Plains, means values for %Ndfa of pea was 55 %, for lentil was 60 %, and for desi and kabuli chickpea was 55 % (Walley et al., 2007). Similarly, %Ndfa for pea, chickpea and lentil was 56, 50, and 60, respectively, in a study conducted in semi-arid Australia (Jensen et al., 2010). The values reported in the current study for seed are comparable to the literature, but for straw are above the average and may have been overestimated. Differences in seasonal N accumulation patterns of pulse crops and reference crop under field conditions and differences in root distribution can lead to erroneous estimates of BNF (van Kessel and Hartley, 2000). The majority of the fields in the 2018 study were carefully managed to suppress weeds, which meant some weeds collected as the reference in the %Ndfa calculation were not a comparable age to the pulse crop and may not have accumulated the same amount of N.

Aboveground N was affected by site and stubble independently. Between sites ABG-N was highest at Davidson, followed by Springside, then Biggar, Central Butte, Theodore, and lastly Indian Head and Swift Current. Aboveground N averaged across sites was higher in pulse crops grown on cereal stubble than on oilseed stubble. The underlying trend where measurements of ABG-N were higher in pulse crops grown on oilseed stubble follows that of MB-C and total PLFA biomarkers for Davidson and Springside, but not for Theodore. For all other sites, the trend in ABG-N more closely follows the pattern in seed and straw yield than it does BNF; as yield decreases, so does ABG-N. Aboveground N may be underestimated in the current study as some

plant material may have been lost to threshing processes or to unrecovered leaves that dropped throughout the growing season (Liu et al., 2019). Typical values of N fixed from BNF of pulse crops range from 10 to 40 kg N ha^{-1} for chickpea (Kyei-Boahen et al., 2002; Walley et al., 2005), 50 to 75 kg N ha^{-1} for lentil (Bremer et al., 1988; Cowell et al., 1989; Matus et al., 1997) and 40 to 86 kg N ha^{-1} for field pea (Beckie and Brandt, 1997; Matus et al., 1997; Soon and Arshad, 2004).

Estimates of fixed N for Swift Current, Davidson, Indian Head, and Theodore are comparable to literature averages; however, the estimates for Central Butte, Biggar, and Springside are higher than average. All three of these sites are situated in different soil zones, therefore disproving the notion that BNF and N acquisition parameters may be predicted by soil zone. Fixed N was higher in pulse crops grown on cereal stubbles at all locations except Davidson and Theodore, which were higher in pulse crops grown on oilseed stubble. Nitrogen derived from soil was affected by an interaction between site and stubble but was variable between sites and stubbles. The amount of N derived from soil was higher in oilseed stubble at Swift Current, Biggar, Indian Head, and Springside. In the case of Swift Current, the amount of N derived from soil was more than the amount of N fixed, suggesting the crops at Swift Current relied more on N from soil than %Ndfa. When inorganic N levels are sufficient or exceed crop N requirements, little BNF occurs, irrespective of other factors (van Kessel and Hartley, 2000). Swift Current did have high nitrate-N content (~60 kg N ha^{-1}) in the 0- to 30-cm depth in the cereal stubble; higher than that at Central Butte.

3.6.3 Conclusion

In summary, BNF of pulse crops was lower when grown on oilseed stubble than cereal stubble except at Biggar in 2017 and Davidson and Theodore in 2018. Soil properties that may have affected BNF were identified and include: varying levels of inorganic N content and P-

deficiency at all sites except Swift Current and in one cereal stubble field at Biggar. A persistent pattern was observed in MB-C and PLFA results where levels of MB-C and total PLFA biomarkers were higher in soil from pulse crops grown on oilseed stubbles at Davidson and Theodore. Hot, dry conditions in 2018 may have affected plant growth and BNF. Unforeseen disease pressures such as *Aphanomyces euteiches* at Wilkie in 2017 and ascochyta blight pressure at Swift Current may also have affected BNF.

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4 ESTIMATING BIOLOGICAL NITROGEN FIXATION AND N-MINERALIZATION POTENTIAL UNDER CONTROLLED ENVIRONMENT CONDITIONS

4.1 Preface

This chapter has two purposes, the first is to confirm field research findings from the previous chapter, by conducting a controlled environment study with yellow pea grown on oilseed and cereal stubbles in soils from the Brown and Black soil zones, measuring the same parameters of BNF. The second purpose is to examine the capacity for the same soils to provide ammonium and nitrate at pre-seeding and at the time of peak BNF of yellow pea. The results from this chapter should provide more insight into the effects of site and stubble on BNF and if ammonium and nitrate potentially provided from each stubble may affect BNF.

4.2 Abstract

Intensive cropping systems have relied on synthetic N fertilizer in the past to satisfy N requirements of cereal and oilseed crops. Including one or more pulse crops in rotation has become a popular option because of their unique ability to biologically fix N in symbiosis with rhizobia bacteria. Mixed results of rotation effects on a pulse crops' ability to fix N were reported from studies at Scott, SK, Swift Current SK, and from a greenhouse experiment using soils from Central Butte, SK. Inorganic N may affect pulse crop BNF and some inorganic N may be supplied through microbial decomposition of previous crop residues. A controlled environment experiment was conducted using soils from the Brown and Black soil zones and two different stubbles, canola and wheat. The experiment had multiple objectives: the first was to estimate BNF of field pea in the

controlled environment, the second was to determine if soil microbial communities differed under each stubble and soil type, and the third, was to examine the N-mineralization and nitrification potential of each stubble in each soil before and after field pea was grown over 48 h. It was hypothesized that BNF and the microbial community would be negatively affected by oilseed stubble and that stubbles would release different rates of ammonium and nitrate into soil. Soil and stubble did not affect BNF, however soil affected N acquisition parameters and yield, with higher levels in pulse crops grown in Black soil. Soil and stubble affected microbial functional group biomarkers independently, which were higher in soil from pulse crops grown on oilseed stubble. In general, gross mineralization rates increased between 3.5 h and 24 h, then decreased between 24 h and 48 h in the pre-field pea assay and increased between 24 h and 48 h in the post-field pea assay. Gross nitrification rates remained negative in both assays but showed an upward trend in both assays over the 48 h. Stubble did not affect gross mineralization or nitrification rates in either assay. The effects on BNF, microbial functional group biomarkers, and N-mineralization potential appear to be driven more by soil than by stubble.

4.3 Introduction

Pulse crops may improve N availability to subsequent crops through the decomposition of N-rich crop residues or due to an N-sparing effect where soil N is conserved for the next crop (Herridge et al., 1995). Increasing the N input from pulse crops to subsequent crops depends on whether the plant is obtaining most of its N from BNF or from soil N (van Kessel and Hartley, 2000). The amount of BNF of a legume is not only determined by legume genotype and rhizobia, but also depends on the interaction between plant-available soil N and legume growth (Unkovich

and Pate, 2000). Previous pulse crop research has primarily examined the benefit of BNF to a succeeding crop, not how a previous crop affects BNF.

There is potential for a previous crop's residue to contribute inorganic N to a pulse crop. Determining if BNF is hindered due to inhibitory effects of inorganic N from mineralization of previous crop residue was an objective identified by Knight (2012). Studies have demonstrated a negative correlation between BNF in pulse crops and available soil N (Salvagiotti et al., 2008; Schipanski et al., 2010). Alternatively, very low concentrations of inorganic N can increase BNF (Gan et al., 2004). Thus, soil mineral N and BNF may be complementary in meeting the N requirements of a legume crop (Hossain et al., 2016). Previous crop residue will affect soil microbial composition as the rate of decay and the amount of nutrient released to soil may depend on crop species (Gan et al., 2011b). Gross rate mineralization and subsequent nitrification measurements provide estimates of the total release of mineral N from a given pool (Bedard-Haughn et al., 2013). Determining whether or not the inhibition of BNF is due to the inhibitory effects of inorganic N from mineralization of a previous year's residue requires further investigation (Knight, 2012).

The overall objective of this study was to examine the effect of rotation on BNF. The objectives of this study were to confirm field study estimates of BNF under controlled conditions, to examine how microbial communities may differ under oilseed and cereal stubbles, and to examine the capacity for each microbial community to provide ammonium and nitrate from preceding crop residues to a pulse crop. To address these objectives, a controlled environment experiment, and two isotope pool dilution assays were performed.

4.4 Materials and Methods

4.4.1 Soil collection, preparation and stubble growth

In summer of 2018, approximately 100 kg of soil was collected at a depth of 15 cm from cereal stubble at Central Butte, SK (Brown soil zone) and Theodore, SK (Black soil zone). Subsamples were used to determine gravimetric moisture content and water holding capacity. Soils were air-dried and coarsely sieved to remove any rocks and to homogenize soil in preparation for planting. Two kg of soil was placed into 4 L plastic pots with 24 pots containing soil from the Brown soil zone, and 24 containing soil from the Black soil zone. Pots were watered to 80% field capacity based on weight for one week and were left to stabilize before planting. For each soil, canola was grown in 12 of the pots with a target plant density of two plants per pot, and wheat was grown in the other 12 pots, with a target plant density of five plants per pot. Prior to planting, fertilizer was applied at rates of 195.2 mg kg^{-1} soil of urea and 20 mg kg^{-1} soil of monoammonium phosphate for wheat and canola, with 20 mg kg^{-1} soil of potassium sulfate applied to canola only. Plants were watered to approximately 70% field capacity, every one to two days.

Canola and wheat residues were collected when plants were near physiological maturity. Cut off stubble was coarsely cut, divided evenly, and spread on top of their respective pots. Pots were then covered and stored outside in cold temperatures (-10 °C) for approximately 5 weeks, then frozen at -20 °C for 2 weeks to simulate a winter freezing period. To regulate temperature and microbial activity, pots were thawed and placed in a growth chamber 2 weeks prior to beginning the experiments. Pots were watered to 50% field capacity for one week after thawing, and field pea was planted the following week.

4.4.2 Estimating biological nitrogen fixation under controlled conditions

4.4.2.1 Experimental setup

A growth chamber experiment was completed to determine the quantity of BNF of CDC 'Meadow' yellow peas using the ^{15}N enriched isotope dilution technique (Hardarson and Danso, 1993). A two-way factorial on a completely randomized design was used. For each soil, 12 field pea seeds (cv. CDC 'Meadow') per pot were planted into canola and wheat stubble in replicate (n = 4). A total of 16 pots were used, four each of canola stubble and wheat stubble from each soil. Prior to seeding the field pea, peat-based inoculant containing *Rhizobium leguminosarum bv. viceae* (Nodulator XL Peat, BASF, Mississauga, ON) was applied by first wetting seed with deionized water, then coating the seed, according to manufacturer's recommendation with an equivalent of 1 x10^9 *rhizobia* per gram. Twelve wheat seeds (unknown cultivar) per pot were seeded at the same time as field pea in duplicate to serve as the non-N_2-fixing reference plants. A total of eight pots of wheat were used. Plants were thinned to 6 plants per pot after germination. All plants were enriched 30 days after planting with 10.1 atom % excess ^{15}N-labeled urea dissolved in deionized water. Fertilizer solution was applied at a rate of 5.6 kg ha^{-1}. Soil was held at ~70% field capacity by weight and corresponding plants were grown for 10 weeks in a growth chamber with a day/night temperature of 24 °C / 21 °C and day/night length of 16 h / 8 h.

4.4.2.2 Plant and soil sampling and analysis

Plants were harvested 70 days after planting, when field pea was beginning podding stage and heads were emerging from the boot in wheat. Plants were air-dried, weighed for above-ground biomass, and ground whole. Above-ground plant samples were analyzed for atom%^{15}N content using a Costech Elemental Combustion system coupled to a Delta V Advantage Mass Spectrometer (Isomass Scientific Inc., Calgary, AB).

After completion of plant growth, and at the same time as plants were harvested, pots were dismantled and soil was sampled from around field pea roots, using sterile technique, for PLFA analysis. Collected soils were sieved to 2 mm and stored in a -80°C freezer until PLFA extraction. Phospholipid fatty acid analysis procedure followed that of section 3.4.7 in Chapter 3.

4.4.2.3 Calculations and statistical analysis

Yield was calculated from total aboveground biomass per pot area. Nitrogen yield was calculated by multiplying % N by total yield. Percent Ndfa was calculated as follows:

$$\%Ndfa = \left[1 - \frac{atom\%^{15}Nexcess_{fixing}}{atom\%^{15}Nexcess_{non-fixing}}\right] x\ 100 \qquad \text{(Eq. 4.1)}$$

where atom % ^{15}N excess refers to the ^{15}N content of the sample minus the background of 0.36637 found in N_2 (Hardarson and Danso, 1993). Amount of N fixed was calculated for the whole plant (Hardarson and Danso, 1993) as:

$$Nfixed = \frac{\%Ndfa\ x\ totalN_{fixing}}{100} \qquad \text{(Eq. 4.2)}$$

Total N acquired from the soil was calculated by subtracting N acquired through BNF from total N in the plant (Knight, 2012).

Statistical analysis was completed using SAS 9.4 (SAS Institute, Cary, NC, USA). Prior to analysis, outliers were identified using box plots and were excluded from the data sets if an observation was more than two standard deviations from the mean. Data were analyzed as a completely randomized design (CRD) using a two-way ANOVA using the PROC GLIMMIX procedure with a significance level of 0.05. The GLIMMIX procedure accounts for normality and variances in the data. The two factors in the ANOVA were soil type and stubble and were considered fixed. There were two soil types, from the Brown soil zone and Black soil zone, and two stubbles, canola and wheat. The RANDOM statement with a RESIDUAL effect was used to

model residual heterogeneity using soil type as the error term. When an effect was significant, the LSMEANS statement was used to facilitate means comparisons.

4.4.3 Determining gross mineralization and nitrification rates using isotope pool dilution

4.4.3.1 Experimental setup

To determine N mineralization and nitrification rates in canola and wheat stubble under Brown and Black soils, the isotope pool dilution (IPD) technique was used. Both soils were assayed for N mineralization and nitrification twice; after canola and wheat were grown (PRE-NOD) and after field pea was grown on the canola and wheat residue soils (POST-NOD). Three pots from each soil and stubble, without field pea were used in the first assay (PRE-NOD). Triplicate pots of each soil and stubble were assayed PRE-NOD and POST-NOD. After harvesting the canola and wheat and simulating winter freezing conditions, the field pea was seeded into pots containing the different residues. Field pea was grown to the podding stage and was harvested. This soil was used in the POST-NOD IPD assay. Over the course of 48 h, assays were stopped at 0 h, 0.25 h, 3.5 h, 24 h, and 48 h. The following method was used for both assays.

4.4.3.2 Isotope pool dilution assay method (Braun et al., 2018)

Soils were adjusted to 50% water holding capacity. Pots were dismantled prior to assays, soil was homogenized by hand and for POST-NOD pots, field pea roots were removed. A subsample of soil was used to determine initial ammonium and nitrate levels using a KCl extraction (Carter and Gregorich, 2008), analyzed on a Technicon Autoanalyzer (SEAL Analytical, Mequon, WI). The IPD protocol involves extracting and measuring ammonium and nitrate five times over 48 h. For each sample time, triplicate 10 g samples of soil were placed into 60 dram vials then covered with perforated parafilm. A total of 60 vials were prepared for each assay: time period (five), soil zone treatment (two), and stubble treatment (two), replicates (three).

Ninety-eight atom % ^{15}N-labeled urea dissolved in deionized water was applied to soil in multiple drops at a rate of 2 µg ^{15}N g^{-1} soil (Di et al., 2000). The rate of 2 µg ^{15}N g^{-1} soil ensured the product pool was increased as little as possible, so that disruption of existing soil N dynamics were avoided but also ensuring sufficient enrichment of the ammonium pool for measurement (Davidson et al., 1991, Di et al., 2000). Samples were shaken by hand to ensure a homogenous mixture. Soils were incubated at 20 °C in the dark for the given time points. At each time point triplicate samples from each soil/stubble treatment were extracted with 100 mL 2 M KCl solution (Carter and Gregorich, 2008). Approximately half of the KCl extract was analyzed on a Technicon Autoanalyzer (SEAL Analytical, Mequon, WI) to determine ammonium and nitrate concentrations. The remainder of the KCl soil extract was diffused onto acidified diffusion disks using a method adapted from Stark and Hart (1996) in a two-step process that first diffuses NH_4^+, then diffuses NO_3^-. Forty mL of the KCl extract was quantitatively transferred into a 60 mL Nalgene bottle and 0.4 g magnesium oxide (MgO) was added. A polytetrafluoroethylene (PTFE) encased acidified diffusion disk was added and the bottle was immediately capped and shaken. Disks were made by placing a 7 mm hole-punched piece of Whatman 1 filter paper (Sigma-Aldrich, St. Louis, MO), pre-rinsed with 2 M KCl solution, then rinsed with deionized water, then dried, onto a strip of PTFE tape. The disk was acidified with 10 µL 2.5 M potassium bisulfate ($KHSO_4$). Another strip of PTFE tape was placed over top and the disk was enclosed by sealing the tape together, forming a circle. Bottles were checked daily to ensure the diffusion disk was not sticking to the side of the bottle and were shaken by hand daily for 8 d. On the eighth day, diffusion disks were taken apart, rinsed in 0.5 M HCl, then in deionized water. Diffusion disks were dried at 60 °C for approximately 10 min. After removing diffusion disks for NH_4^+ diffusion, bottles were left uncapped for 4 d, swirling each day, to eliminate any residual NH_4^+. With the same KCl

extracted sample, the process was repeated with new disks and with 0.4 g of Devarda's alloy in place of MgO over a period of 6 d. Standards for NH_4^+ and NO_3^- were prepared separately for each diffusion step at concentrations of 0.5 ppm, 1 ppm, 2 ppm, 3 ppm, 5 ppm, 7.5 ppm and 10 ppm, respectively. Dried diffusion disks were encapsulated and analyzed using an Isotope Ratio Mass Spectrometry to quantify ^{15}N-labeled ammonium and nitrate contents.

4.4.3.3 Calculations and statistical analysis

Gross rates of mineralization and nitrification were calculated according to Hart et al., (1994) as follows:

$$m = \frac{[NH_4{}^+]_0 - [NH_4{}^+]_t}{t} * \frac{log(APE_0/APE_t)}{log\left([NH_4{}^+]_0/[NH_4{}^+]_t\right)} \qquad \text{(Eq. 4.3)}$$

$$c_A = m - \frac{[NH_4{}^+]_t - [NH_4{}^+]_0}{t} \qquad \text{(Eq. 4.4)}$$

where m is the gross mineralization rate, c_A is the NH_4^+ consumption rate, t is time, APE_0 is the atom % excess of NH_4^+ pool at time 0 and APE_t is the atom % excess of NH_4^+ pool at time-t (where t = 0.25 h, 3.5 h, 24, h, and 48 h), $[NH_4^+]_0$ is the total NH_4^+ concentration at time 0, and $[NH_4^+]_t$ is the total NH_4^+ concentration at time-t. Background enrichments are assumed to be 0.3663 atom % ^{15}N. To calculate the gross nitrification and consumption rates the same equations were used, substituting NO_3^- concentrations and atom % ^{15}N enrichments, and where "m" becomes "n", and c_A becomes c_N.

Data were statistically analyzed using SAS software (SAS Institute, Inc., version 9.4, Cary, NC). Before analysis, outliers were identified using boxplots and were eliminated from the data set if an observation was more than two standard deviations from the mean. One replicate was identified as an outlier but was not removed because all three replicates are required for ANOVA. Data were analyzed as a completely randomized design and were subjected to a three-way

ANOVA using the PROC GLIMMIX procedure with a significance level of 0.05. The GLIMMIX procedure accounts for normality and variances. Soil type, stubble, and time were considered fixed effects. Measurements for each time point were grouped together. The RANDOM statement with a RESIDUAL effect was used to model residual heterogeneity using soil as the error term. When an effect was significant, the LSMEANS statement was used to facilitate means comparisons.

4.5 Results

4.5.1 Estimating biological nitrogen fixation under controlled conditions

Soil, stubble, or an interaction between the two factors did not affect %Ndfa in the growth chamber experiment (Table 4.1). Aboveground N, fixed N, and Ndfs were affected by soil ($p<0.05$) (Table 4.1). Aboveground N of field pea was higher in Black soil (33 g pot^{-1}) than in Brown soil (19 g pot^{-1}) ($p=0.0033$) (Table 4.1). The amount of N fixed was higher in Black soil (28 g pot^{-1}) than in Brown soil (16 g pot^{-1}) (Table 4.1). Nitrogen derived from soil was also higher in the Black soil (6 g pot^{-1}) than in Brown soil (4 g pot^{-1}). Biomass of each pot was affected by soil ($p=0.0001$) and stubble ($p=0.0200$) (Table 4.1). Biomass in Black soil was 17 g pot^{-1} and in Brown soil was 12 g pot^{-1}. Biomass of field pea grown on cereal stubble was 16 g pot^{-1} and on oilseed stubble was 14 g pot^{-1} (Table 4.1).

Table 4.0.1. Nitrogen acquisition of CDC 'Meadow' yellow field pea grown on oilseed or cereal stubbles in soil from the Brown and Black soil zones under controlled environment conditions.

Soil Zone	Stubble	%Ndfa	ABG-N[†]	Fixed N	Ndfs	Biomass
Main effects		%	-------------------------	g pot^{-1}	-------------------------	
Brown		81a[‡]	19b	16b	4b	12b
Black		83a	33a	28a	6a	17a
	OIL	81a	27a	22a	5a	14b
	CER	83a	26a	22a	4a	16a
Interactions						
Brown	OIL	80a	20a	16a	4a	11c
Brown	CER	82a	19a	16a	3a	14bc
Black	OIL	82a	34a	28a	6a	16ab
Black	CER	85a	32a	27a	5a	18a
-------------------------			*Probability*	-------------------------		
Soil		0.0617	**0.0033**	**0.0026**	**0.0187**	**0.0001**
Stubble		0.0973	0.6979	0.8606	0.1752	**0.0200**
Soil*Stubble		0.8241	0.8860	0.9093	0.7798	0.8928

[†] ABG-N = aboveground nitrogen, Ndfs = nitrogen derived from soil, %Ndfa = percentage of N derived from atmosphere, OIL = oilseed stubble, CER = cereal stubble
[‡] Values are means (n = 4). Means followed by the same letter are not significantly different. Bolded values indicate significance at p = 0.05.

4.5.2 Soil microbial community

Soil affected gram positive, gram negative, actinobacteria, and AMF functional group abundance and the total amount of biomarkers present (p<0.0001); all levels of PLFAs were higher in the Black soil (Table 4.2). The ratio of fungi to bacteria was also affected by soil (p=0.0002) but was higher in the Brown soil (Table 4.2). The largest difference was observed in total PLFA biomarkers where the Black soil had 48 nmol g^{-1} soil more than the Brown soil.

Stubble type affected gram positive, gram negative, actinobacteria, and other fungal biomarker abundance (p<0.05) as well as the total amount of biomarkers (p=0.0077) (Table 4.2). Oilseed stubble had slightly higher abundance of PLFAs in each affected functional group (Table 4.2). The largest difference between the two stubbles was in total PLFA biomarkers with oilseed stubble having 13 nmol g^{-1} soil more than cereal stubble.

Table 4.0.2. PLFA functional group biomarker content from bulk soil of CDC 'Meadow' yellow field pea roots, grown in soil from the Brown and Black soil zones in controlled environment conditions, sampled at podding stage.

Soil	Stubble	G^{+}†	G^{-}	ACT	AMF	FUN	Total	F:B
Main effects		-------------------------------	----------	nmol g^{-1} soil	----------	------------------------------		
Brown‡		14b‡	22b	8b	3b	3a	74b	0.11a
Black		24a	39a	13a	5a	2a	122a	0.07b
	OIL	20a	33a	11a	4a	3a	104a	0.10a
	CER	18b	29b	10b	4a	2b	91b	0.09a
Interactions								
Brown	OIL	15b	24b	8b	3b	3a	79b	0.12a
Brown	CER	13c	20b	7c	3b	2b	69b	0.11ab
Black	OIL	26a	41a	14a	5a	3ab	130a	0.08bc
Black	CER	22a	37a	13a	5a	2b	114a	0.06c
----------	----------	----------	----------	*Probability*	----------	----------	----------	----------
Soil		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.2718	**<0.0001**	**0.0002**
Stubble		**0.0088**	**0.0142**	**0.0069**	0.4783	**0.0041**	**0.0077**	0.1957
Soil*Stubble		0.6960	0.7470	0.5363	0.3287	0.3789	0.4979	0.4626

† G^{+} = gram positive bacteria, G^{-} = gram negative bacteria, ACT = actinobacteria, AMF = arbuscular mycorrhizal fungi, ACT = actinobacteria, FUN = fungi, F:B = fungi to bacteria ratio (unitless), OIL = oilseed stubble, CER = cereal stubble

‡ Values are means (n = 4). Means with the same letters in a column are not significantly different. Bolded values indicate significance at $p = 0.05$.

4.5.3 Gross mineralization and nitrification rates

In the PRE-NOD assay, time affected gross mineralization and nitrification rates ($p<0.0001$) (Table 4.3). Ammonium was immobilized at the 0.25 h and 3.5 h time points and was mineralized at a rate of 0.12 mg NH_4^+ kg^{-1} soil h^{-1} at the 24 h time point and a rate of 0.06 mg NH_4^+ kg^{-1} soil h^{-1} at the 48 h time point, suggesting that the gross mineralization rate peaked at the 24 h time point (Fig 4.1). In the gross nitrification rates, immobilization or denitrification occurred at all time points, as indicated by negative mean values, but increased significantly from 3.5 h to 24 and 48 h (Fig 4.1).

In the POST-NOD assay an interaction between soil and time affected both gross mineralization and nitrification rates (p<0.0001) (Table 4.3). At 0.25 h, less ammonium was produced in the Brown (0.09 mg NH_4^+ kg^{-1} soil h^{-1}) soil than in the Black (0.14 mg NH_4^+ kg^{-1} soil

h^{-1}) (Fig. 4.2). Mineralization rates went up by half in the Brown soil (0.18 mg NH_4^+ kg^{-1} soil h^{-1}) and by one third in the Black soil (0.21 mg NH_4^+ kg^{-1} soil h^{-1}) at 48 h. Similar to the PRE-NOD assay's gross nitrification rates, negative values indicate immobilization or denitrification of nitrate occurred more in soil from the Black soil zone than the Brown (Fig. 4.2) at 0.25 h but was not different at the 3.5 or 24 h and 48 h times. Both assays were not affected by stubble.

Table 4.3. Results of ANOVA for isotope pool dilution assay gross mineralization and nitrification rates in oilseed and cereal stubble soils, where no field pea was grown (PRE-NOD) and after nodulation of CDC 'Meadow' yellow field pea occurred (POST-NOD), measured at times 0.25 h, 3.5 h, 24 h, and 48 h.

Effect	*df*†	Gross Mineralization	*df*	Gross Nitrification
---Probability (PRE-NOD Assay)---				
Soil (SOIL)	1	0.0814	1	0.4969
Stubble (STU)	1	0.7581	1	0.5639
Time (T)	3	**<0.0001**‡	3	**<0.0001**
SOIL*STU	1	0.3505	1	0.5639
SOIL*T	3	0.3501	3	0.8745
STU*T	3	0.7606	3	0.8716
SOIL*STU*T	3	0.3758	3	0.8507
---Probability (POST-NOD Assay)---				
Soil (SOIL)	1	**<0.0001**	1	**<0.0001**
Stubble (STU)	1	0.1814	1	0.8884
Time (T)	3	**<0.0001**	3	**<0.0001**
SOIL*STU	1	0.2270	1	0.0552
SOIL*T	3	**<0.0001**	3	**<0.0001**
STU*T	3	0.2480	3	1.0000
SOIL*STU*T	3	0.3518	3	**0.0437**

†df = numerator degrees of freedom
‡Bolded values denote significant difference ($p = 0.05$)

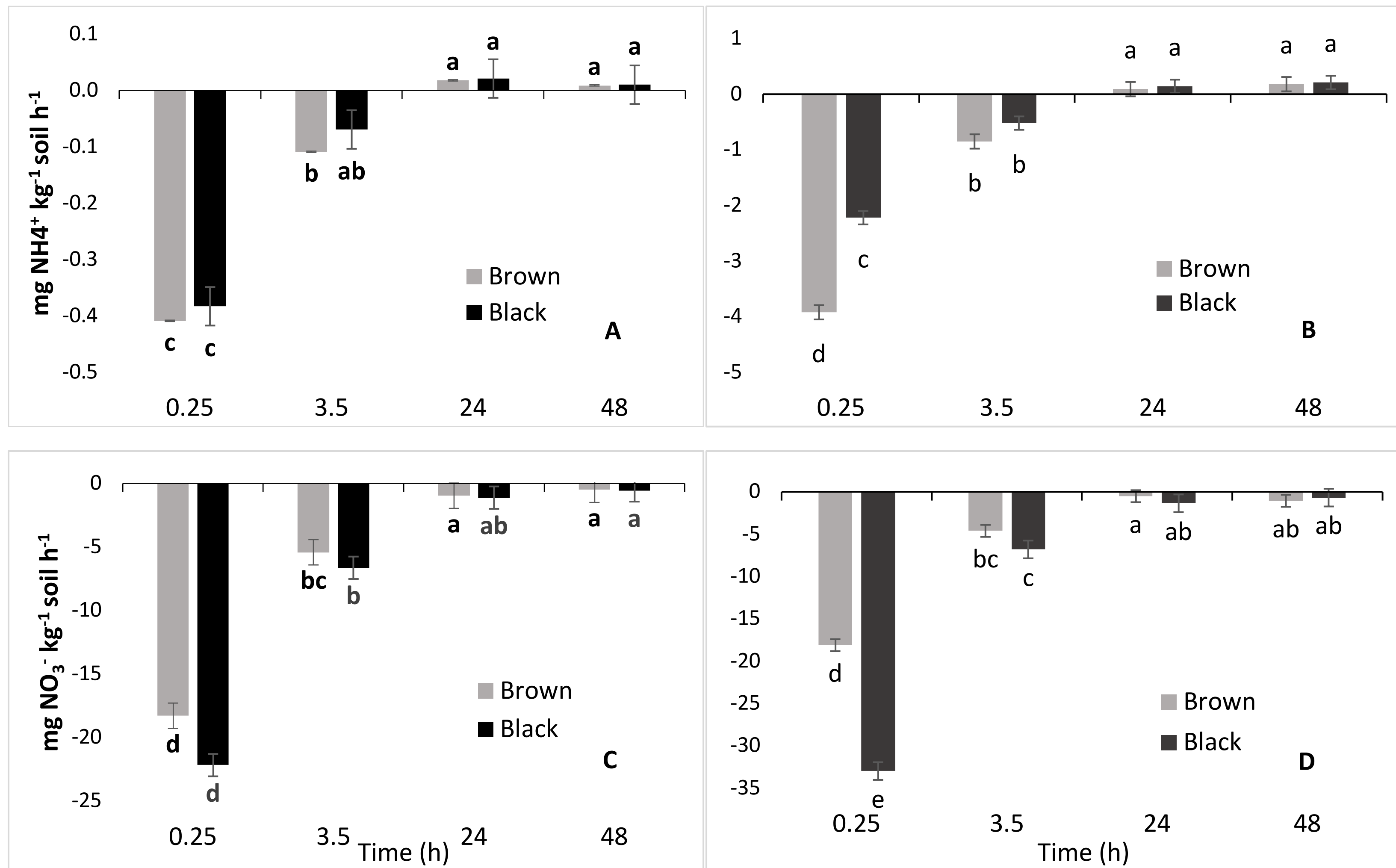

Fig. 4.1 Effect of soil type (Brown or Black) and time (0.25 h, 3.5 h, 24 h, and 48 h) on gross mineralization (top) and nitrification (bottom) rates before field pea (A and C) and after field pea (B and D) was grown. Treatment bars with the same letters above or below are not significantly different ($p<0.05$).

4.6 Discussion

4.6.1 Biological nitrogen fixation in controlled conditions

The purpose of estimating BNF in a controlled environment was to gain more insight into the potential effect of oilseed or cereal stubble on BNF. Variability in %Ndfa is expected under field conditions and it is well recognized that BNF is sensitive to numerous environmental and edaphic factors (Walley et al., 2007). By providing the same growing condition for the field pea in Black and Brown soil, the environmental effect on BNF may be reduced. In the current study, no effect of soil, stubble, or an interaction between the two on BNF was found. Estimates of %Ndfa of field pea in grain were between 55 and 66% (Walley et al., 2007). The current study's estimates of %Ndfa are for the whole plant, excluding roots, harvested at the beginning of podding, which may be partially responsible for values that are higher than estimates in the literature. The ample light and water provided, and favourable temperature in the controlled environment is likely to be the cause of higher %Ndfa estimates. Soil type affected ABG-N, N fixed, and Ndfs independently. Even though BNF was not directly affected by soil or stubble, it appears that N acquisition may be affected more by soil than by stubble. Field pea biomass was affected by soil and stubble independently and was higher in the Black soil and in field pea grown on cereal stubble. The current study's results are not congruent with results from a greenhouse study that used soil from Central Butte, where pulse crops following wheat had higher amounts of total N, amount of N fixed, and Ndfs than following canola (Chen, 2016). The soil cores used in the controlled environment of the previous study were intact, and therefore management history of the soil was retained; however, soil in each core was inherently heterogeneous; inorganic N may have varied between cores due to previous crops, and biomass between plants in the same cores varied (Chen, 2016). Soils from the current study were collected from the 0- to 15-cm depth and were

homogenized to reduce variability in preparation for planting canola and wheat for stubbles before growing field pea. The soil cores from the previous study and volume of soil from the current study were approximately the same size; N uptake patterns should theoretically be the same because plants are exploiting the same volume of soil in each study (Unkovich et al., 2008).

4.6.2 Soil microbiological effects

Phospholipid fatty acid analysis is a method commonly used to study microbial community structure and abundance and was used to characterize microbial communities in this study. This method is robust due to its relative ease of extraction, cost effectiveness and sensitive and reproducible results (Frostegård et al., 2011). Microbial biodiversity of a rotation sequence may make a soil more flexible in responding to environmental and/or biotic fluctuations and differences in BNF may be related to differences in microbial populations among the different rotations (Knight, 2012). Soil and stubble each affected soil microbial functional group biomarkers independently. Field pea grown on oilseed stubbles in both soils and in black soil had higher levels of functional group biomarkers. Gram positive, gram negative, actinobacteria, and fungi biomarkers were higher in field pea grown on oilseed stubble than in cereal stubble. These functional group biomarkers may not have an impeding effect on BNF of field pea, as the difference in BNF between the two stubbles was very little. Enzyme activities and microbial biomass, as indicated by PLFA biomarkers were positively related to field pea biomass production, with the exception of gram negative biomarkers, which had a positive relationship with plant micronutrient content and was inversely related to root abundance (Nayyar et al., 2009). Gram negative biomarkers were highest in both stubbles and soils.

4.6.3 Gross mineralization and nitrification rates

Gross processes of mineralization, nitrification, and immobilization may occur simultaneously in the soil and their relative magnitudes will determine if there is a net release of N into the soil (Recous et al., 1999; Murphy et al., 2003). Estimates of gross N fluxes may help in predicting N availability in the soil, in particular when these measures of inorganic N production are repeated throughout the growing season, providing a better idea of when maximum mineralization occurs (Bedard-Haughn et al., 2013). It is useful to measure gross mineralization and nitrification at the PRE-NOD and POST-NOD stages to understand if stubble has an effect on the supply of inorganic N to pulse crops pre-seeding, and at the approximate time of peak nodulation. A higher soil C:N ratio from greater inputs of crop residues with higher C:N ratios causes greater immobilization of N (Powlson and Barraclough, 1993).

Stubble type did not affect gross mineralization or nitrification rates in either the PRE-NOD or POST-NOD assays. Time affected the PRE-NOD assay gross mineralization and nitrification rates and an interaction between soil and time affected the POST-NOD assay. Gross mineralization rates in both soils and both stubbles peaked at the 24 h mark in the PRE-NOD assay. In the POST-NOD assay, gross mineralization increased from 3.5 h to 24 h, with an upward trend into the 48 h mark. Mineralization rates in the post-field pea assay between 24 h and 48 h doubled in the Brown soil and increased by one third in the Black soil. Gross nitrification rates remained negative throughout the assays, but there appears to be a positive upward trend in the Black soil in the POST-NOD assay. Gross processes of mineralization, nitrification and immobilization occur simultaneously in soil and their relative magnitudes will determine whether there is a net release of N into the soil (Recous et al., 1999; Murphy et al., 2003). In both assays, a release of ammonium occurred; however, in the POST-NOD assay, more ammonium was released at 24 h and 48 h than in the PRE-NOD assay at 24 h. The POST-NOD assay may have started with more inorganic N in

soil due to labile root-derived organic N compounds that were mineralized (Janzen, 1990) or inorganic N that was released directly from roots (Brophy and Hiechel, 1989). Inorganic N comprised 4.1 %, 12.2 % and 13 % of total N rhizodeposition in soil cropped with canola, N-fertilized pea and non-fertilized pea, respectively (Arcand et al., 2013). Furthermore, soil microbial community may be influenced by rhizodeposition of pulse crop roots because soil microbes utilize rhizo-deposits as a C source (Bais et al., 2006).

The assumptions that must be met during an isotope pool dilution study are: microorganisms do not discriminate between ^{15}N and ^{14}N, rates of processes measured remain constant over the incubation period, and ^{15}N assimilated during the incubation period is not re-mineralized (Kirkham and Bartholomew, 1954). Negative nitrification values from a previous isotope pool dilution study concluded that they most likely represented a violation of one or more assumptions of the isotope pool dilution method: the violation of immobilization and re-mineralization assumption, where the added ^{15}N is re-mineralized within the 24 h period (Bedard-Haughn et al., 2013). Regardless of violations of experimental assumptions, the isotope pool dilution method remains the most accessible means for determining gross mineralization and nitrification rates (Booth et al., 2005).

4.6.4 Conclusion

This study provided estimates of BNF under controlled environment conditions. Biological nitrogen fixation was not affected by soil or stubble type. Soil type affected N acquisition parameters and yield; ABG- N, fixed N, Ndfs, and biomass were higher in Black soil than Brown soil. Biomass was also affected by stubble and was higher when grown on cereal stubble than on oilseed stubble. Soil and stubble affected microbial functional group biomarkers independently. Higher levels of functional group biomarkers were found in soil from field pea grown on oilseed

stubble as well as from that grown in both stubbles in black soil. Stubble did not affect gross mineralization or nitrification rates in either the PRE-NOD assay or the POST-NOD assay. Soil and timing of the assay (PRE or POST) affected gross mineralization and nitrification rates and patterns. In the POST-NOD assay ammonium production between 24 h and 48 h doubled in the Brown soil and increased by one third in the Black soil.

4.7 References

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5 SYNTHESIS AND CONCLUSIONS

5.1 Overview

As N is the most limiting factor for crop production on the prairies, synthetic N fertilizer production and use has risen, and continuous cropping of non-legume annual crops has become dependent on it. However, rotations that include pulse crops offer an alternative to synthetic N use in traditional cereal and oilseed-based rotations because of their unique ability to symbiotically fix N_2 with the aid of rhizobia bacteria. Pulse crops may also supply N-rich residues to subsequent crops depending on their BNF capability, potentially reducing fertilizer inputs (Gan et al., 2011a) and decreasing the carbon footprint of the agricultural system (Lemke et al., 2007; Gan et al., 2011c).

Prior research has primarily focused on the N benefit a pulse crop can confer to a succeeding crop, not how a preceding crop may affect BNF of a pulse crop. Previous studies presented conflicting results on the effect of oilseed and cereal stubble on BNF: pulse crop BNF decreased when grown on mustard stubble at Swift Current (Knight, 2015) and also when grown on canola stubble in soils from Central Butte, SK (Chen, 2016), but increased when field pea was grown in rotations with canola at Scott, SK (Knight, 2012).

The research in this MSc thesis examines the effect of oilseed or cereal stubble on BNF of pulse crops and related soil properties in multiple soil zones in Saskatchewan. The results provide field and controlled environment estimates of pulse crop BNF when grown after oilseed and cereal stubbles. Soil properties that may have affected BNF were characterized and were subjectively or statistically related to BNF estimates.

5.2 Summary of findings

Biological nitrogen fixation was negatively affected when grown after an oilseed at all locations except at Biggar, in 2017 and at Davidson and Theodore in 2018. However, BNF was not affected by soil or stubble under controlled environment conditions. Growing season conditions were hotter and drier in 2018 and results from a previous study examining BNF by pulse crops at Swift Current highlight the importance of environmental factors on BNF and yield, particularly the negative effect of low rainfall (Hossain et al., 2016). The experiment from Chapter 4 with controlled environment factors may provide further evidence of the influence of weather on BNF across the Brown, Dark Brown and Black soil zones of Saskatchewan.

A suggestion was made that the rotation sequence effect on BNF may be related to OM levels in the different soil zones and that OM may buffer the microbial populations in soil, making them more resilient to abiotic changes (Knight, 2012). Chen (2016) speculated that higher OM may buffer adverse effects of canola residue's volatile fatty acids and phenolic compounds, that may inhibit seedling growth of a subsequent crop (Wanniarachchi and Voroney, 1997). The canola microbiome is significantly different from other crop microbiomes (Lay et al., 2018) and different plant species often select for different root-associated microorganisms (Hallmann et al., 1997). Canola may also reduce microorganism populations such as rhizobia or AMF because these microorganisms do not colonize canola roots, and populations may therefore decrease (Lay et al., 2018). However, higher BNF in pulse crops grown on oilseed stubble occurred in two different soil zones. In addition, effects of stubble on pulse crop BNF was not consistent across the three soil zones included in the field study. Biological nitrogen fixation in pulse crops grown on oilseed stubble was lower than on wheat stubble in the Brown soil zone at both sites, and it could be

recommended that producers in this area of the province should avoid growing pulse crops on oilseed stubble; however, more site years in this soil zone are necessary to make a firm conclusion.

The higher MB-C in soil from pulse crops grown on oilseed stubble at Theodore, Davidson, and Springside was surprising but was mirrored in the controlled environment experiment where higher amounts of PLFA biomarkers were found in soil from the Black soil zone and in soil from pulse crops grown on oilseed stubble. Long-term management factors may partially explain the MB-C and total functional group biomarkers in the two studies. In continuously cropped, diverse rotation systems, microbial communities are bolstered by a rich spectrum of resources (Drinkwater and Snapp, 2007b). Continuous cropping may serve as an equalizer for long-term development of microbial community structure (Drijber et al., 2000). Furthermore, increased amounts of total microbial biomass were observed in surface soils in no-till soils at four sites across the prairies (Helgason et al., 2009). A comparison of the decomposition and N and P mineralization of canola, pea, and wheat residues showed that wheat straw had the highest C:N ratio, followed by canola straw, then pea straw (Soon and Arshad, 2002). Also, pea and canola straws were found to decompose more rapidly than wheat straws over a period of 10 to 11 months, while the opposite was true for root residues (Soon and Arshad, 2002). High C:N ratios of residues have been associated with increased soil MB-C (Lupwayi et al., 2004). However, in the isotope pool dilution experiment from Chapter 4, stubble did not affect gross mineralization or nitrification rates before or after BNF of the pulse crop. Furthermore, there is conflicting evidence that crop rotations have an effect on soil organic matter and crop management decisions alone are likely to influence microbial and SOM dynamics via their effects on the number of crop rotations, their planting patterns and residue biochemistry (McDaniel et al., 2014).

The MB-C measurements from the 2018 field study were taken after pulse crops had finished flowering when peak BNF occurs. It is possible that functional group biomarker measurements in pulse crops on cereal and oilseed stubbles fluctuate with edaphic properties throughout the growing season (Bainard et al., 2016). Soil moisture content is a strong factor related to temporal shifts in microbial community composition, particularly bacterial community composition. Measurements of MB-C made in spring months when there was more precipitation were very different compared to later sampling periods when soil moisture was low (Bainard et al., 2016). A temporal pattern in microbial community due to precipitation may explain the field study results, but not the controlled environment results. Perhaps, with the breakdown of cereal and oilseed residues, microbial communities fluctuate throughout the growing season and total biomarkers in pulse crops grown on cereal stubbles peaked before the time of sampling.

5.3 Future research

The effect of residue on microbial community could be measured over time while simultaneously growing field pea, lentil, or chickpea, in soil from the Dark Brown and Black soil zones under controlled environment conditions. By measuring microbial community temporally, the effect of residue decomposition and its effect on BNF may be measured throughout the growth of a pulse crop. Coupled with soil microbial activity, oilseed and cereal residue quality regulates the rate and pattern of N mineralization from crop residues (Lupwayi and Kennedy, 2007).

The gross mineralization and nitrification rates from Chapter 4 provide "snapshots" from pre-seeding and at the time when peak nodulation would occur. Determining mineralization and nitrification rates from oilseed and cereal stubble residues, throughout the growth of field pea or other pulse crops will provide a more accurate assessment of ammonium and nitrate release into

soil. This could be done using the isotope pool dilution method, or by an incubation method that does not require the isotope pool dilution technique as the assumptions of the technique are difficult to maintain.

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APPENDICES

Table A.1. Nodulation assessment criteria and scoring adapted from Risula, (n.d.), Saskatchewan Pulse Growers Association.

Assessment Criteria	**Assessment Score**
Plant Growth and Vigour	
Plants green and vigorous	5
Plants green and relatively small	3
Plants slightly chlorotic	2
Plants very chlorotic	1
Colour and Abundance	
>5 clusters of pink pigmented nodules	5
3 to 5 cluster groups of mostly pink nodules	3
<3 clusters of nodules OR white or green nodules	1
No nodules OR white or green nodules	0
Nodule Position	
Both crown and lateral nodulation	3
Mostly crown nodulation only	2
Mostly lateral nodulation only	1
Total Score	
Effective nodulation	11 to 13
Nodulation less effective	7 to 10
Poor nodulation	1 to 6

Table A.2. Generalized critical limits for N, P, K, and S in soils for cereal and oilseed crops from Norwest Laboratory, Edmonton, AB.

	N†	P	K	S
	$kg\ ha^{-1}$			
Deficient	<67	<34	<179	<9
Marginal	67-112	34-56	179-280	9-36
Optimal	112-168	56-134	280-1120	36-90
Excessive	>245	>134	>1120	>90

†N (NO_3^-) and S supply to 0- to 60-cm depth, P and K supply to 0- to 15-cm depth