Below-ground plant residues as a source of nitrogen in double-crop forage systems.

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1.1 Introduction

Nitrogen is recognized as the most limiting nutrient for plant growth in agroecosystems. All major agricultural crops, with the exception of legumes (Fabaceae), rely on inorganic nitrogen (N) supplied by soils for growth. Inorganic soil N may be derived from atmospheric N_2 via biologically fixed, or from N-fertilizer applications. N fertilizer applications have increased worldwide in the past half century, and their use in agricultural commodity production is a significant driver of multiple ecological crisis, including anthropogenic climate change through the gaseous loss of fertilizer N as N_2O (Peoples et al., 1995), and aquatic eutrophication from nitrate leaching (Crews & Peoples, 2004).

Due to these and other negative effects of conventional, high-input agricultural production systems, many agricultural scientists and practitioners advocate for the mitigation or replacement of synthetic N inputs with biological nitrogen fixation by legumes (Crews and Peoples, 2004; McSwiney et al., 2010). Perennial pasture, crop rotations and intercropping legumes with non- N fixing plants are all effective methods of exploiting the N-fixing capacity of legumes to supply N to agricultural soils. Maximizing the advantages of biological nitrogen fixation requires the proper management of legumes and their residues

Most research has focused on above-ground legume biomass as the primary, or even sole source of N in low-input systems. Oftentimes, nitrogen benefit of cover crops cannot be fully accounted for based on N in above-ground residues and recovered root biomass (Chalk, 1998; Khan et al., 2003; Peoples et al., 1995). One explanations for these discrepancies is that many estimates of total below-ground allocations of nitrogen by legumes derived from the recovery of macro-roots significantly underestimate root-derived N (McNeill et al. 1997). Below-ground N can be divided into two pools: living, recoverable roots and the compounds and tissues released by living roots during growth. This former pool, termed rhizodeposition, is heterogeneous and poorly understood, yet quantitatively significant (Jones et al., 2009; Wichern et al., 2008). In cropping systems where above-ground legume biomass are harvested for feed or grain, root-derived N is the dominant source of N to the soil.

Optimal management of nutrient cycling in such cropping systems depends on accurate knowledge of the quantities and transformations of below-ground, plant-derived N (BGN) in both short- and long-term time frames. In the short term, cover crop residue decomposition and N mineralization is frequently non-synchronous with crop N demand, limiting the efficacy of cover-crop based N management (Crews & Peoples, 2005). In the longer term, BGN undergoes multiple

transformations through different pools of soil organic matter, and simple estimates of N mineralization rates are insufficient for understanding impacts of BGN and residue decomposition on these pools of labile soil organic matter.

The quantity of BGN, it's decomposition, distribution in various soil organic matter pools, and ultimate availability subsequent crops depend on many factors, including species composition, biological N_2 fixation, root physical and chemical characteristics, and soil chemistry and mineralogy. The subject of this review are factors that determine the quantity of root- and rhizodeposit- derived N from legume cover crops, and their fate in the soil.

1.2 Roots

Plant roots are an important source of organic matter in the soil. In forage crops, as much as 70% of photo-assimilated carbon (C) can be allocated below-ground. Root biomass can be measured as a shoot-to-root ratio, which is influenced by factors such as plant species and phenology, resource competition, climate, nutrient status and soil depth. Plant species are perhaps the strongest determinant of root biomass. Bolinder et al. (2007) reviewed plant C allocations of common agricultural crops. They reported that shoot-to-root ratios of annual cereals was typically much higher than that of perennial forages. In forages bi-cultures, shoot-to-root ratios of legumes was frequently double that of grasses. Legume root biomass and root length density is also typically lower than that of cereals, as their capacity to fix N from the atmosphere reduces their need to scavenge N from the soil (Jensen, 1996). Nitrogen concentrations in legume roots are typically greater than those in cereal roots, as cereals are limited by N availability, while legumes can acquire through N_2 fixation (Gan et al, 2011). Nitrogen scarcity typically decreases shoot-to-root ratios, as plants prioritize root growth to scavenge for scarcer resources. In one study, root biomass increased in barley fertilized with N, but shoot growth increased even more, resulting in an increased shoot-to-root ratio (Kwabiah et al. 2005). N scarcity also increases N_2 fixation in legumes (Corre-Hellou et al., 2006).

The root biomass of legumes and perennial forages consist of over 70% fine roots (Jani et al., 2015; Rasmussen et al., 2010), with the majority of this biomass occurring in the upper 7 cm of soil (Gan et al., 2011; Gardner & Sarrantonio, 2012; Rasmussen et al., 2010). While roots in general have higher C/N ratios than shoots, fine roots contain greater %N than coarse roots (Bolger et al., 2003; Jani et al., 2015). Nitrogen concentrations are also affected by plant maturity. N decreases in roots as plants mature; unlike C, N is mobile and can be trans-located from roots to shoots during reproductive growth. However, this effect was found to be more significant in cereals than in legumes (Gardener and Sarrantonio, 2012).

Intercropping legumes and cereals can greatly affect root biomass, morphology, and total N contents of plant roots. Legume and cereals occupy different competitive niches, and when grown in proximity, can increase productivity through complementary functions. Cereals compete aggressively for nitrogen, reducing available soil N. In response to this N scarcity, legumes fix more N from the atmosphere; as much as 92% of N may be derived from biological N fixation (Høgh-Jensen & Schjoerring, 2001). Jensen (1996) reported an increase in N-fixation from 62% to 82% of total plant N in inter-cropped pea and barley over mono-cropped pea. Resource competition can also stimulate increased root biomass production. (Erik Steen Jensen, 1996; Kwabiah et al. 2005;). Although inter-specific competition is the main driver of N use efficiency and increase productivity of inter-cropping, N transfer from legumes to cereals is an important function affecting soil N pools, especially in low N environments (Hauggaard-Nielsen & Jensen, 2005). Pathways of N-transfer include fine-root and nodule turnover (Dubach & Russelle, 1994) and rhizodeposition (Høgh-Jensen & Schjoerring, 2001). The relative significance of these pathways is still uncertain, but the degree of N transfer from legumes to cereals inevitably affects total N uptake from soil, and subsequent quantities of N in various pools in soil.

1.3 Rhizodeposition

Rhizodeposition is defined as the soluble and particular organic compounds released by living roots during growth. Rhizodeposition constitutes a substantial input of organic matter into the soil; it has been estimated that 11%-17% of net assimilated plant C is released into the soil as rhizodeposition, which amounts to ~27%-50% of C partitioned below-ground (Jones et al., 2009). Rhizodeposition has been typically been studied exclusively in terms of carbon (Hütsch et al., 2002; Jones et al., 2009; Paterson, 2013), but recent research has focused on rhizodeposition as an important source of nitrogen to soil (Fustec et al., 2010; Wichern et al., 2008). Rhizodeposits can be differentiated according to their physiological origin, solubility, chemical composition, and relative quantity. The particulate sources of rhizodeposits include sloughed root-tip cells, root hair turnover, and fine roots. Sloughed root-cells are a function of root growth, while root-hair turnover occurs continuously throughout root life. Fine roots and root fragments, while not strictly considered rhizodeposits, are often methodologically indistinguishable from other categories of rhizodeposition. Non-particular, soluble sources of rhizodeposits include exudates, mucilage, lysates and ions. Exudates, defined as low molecular-weight substances released by roots, consist of sugars, amino acids and organic acids. They are released both passively and actively, and constitute the single largest source of rhizodeposited C. Mucilage is secreted in the course of root growth, and is composed primarily of sugars and proteins. The

relative quantities of each component of rhizodeposition is uncertain. A review by Nguyen (2003) calculates that root exudates release 10-100 more C than sloughed cells and mucilage, and 3 orders of magnitude more than root hair turnover.

Differences in the composition and origin of rhizodeposits are important for understanding the role of rhizodeposition in soil N cycling. For example, mucilage may consist of up to 6% protein, while concentrations of amino acids in root exudates, ranges from 6%- 31%, depending on plant phenology and species (Gransee & Wittenmayer, 2000; Hütsch et al., 2002). However, Jones et al. (2009) suggest that the low concentrations of amino acids in root exudates implies that a majority of rhizodeposited N comes from root turnover. Given the wide range of estimates, as well as methodological complications of measuring rhizodeposited compounds, %N in rhizodeposits is best measured as an aggregate of multiple root functions (Wichern et al. 2008).

Many biotic and abiotic factors affect the quantity and quality of plant rhizodeposition, including soil texture, CO_2 concentration, water stress, plant species and phenology, root morphology and rhizosphere microbial communities (De Graaff et al., 2007; Van Der Krift et al., 2001). Although many of these factors are poorly understood, the relationship between root biomass and plant phenology is well-established. The primary control on rhizodeposition is the allocation of photo-assimilated C below-ground. As plants mature, less C is allocated to roots, and more remains above-ground, meaning that older plants release fewer rhizodeposits into the soil (Dijkstra e al., 2006; Fu & Cheng, 2002). Similarly, root morphology affects rhizodeposition. Plants species with more fine roots have greater root surface area that comes into contact with soil, resulting in more sloughed border cells, passive exudation, and mucilage secretion.

Abiotic factors such as soil texture and soil nitrogen also affect rhizodeposition. While less C is allocated below-ground in fine-textured soil, rhizodeposition as a proportion of root biomass is encouraged by higher concentrations of clay (Jones et al., 2009; Merckx et al., 1985). Finer texture soils allow more root-soil surface contact, and soluble exudates may bind to clay particles, creating conditions for greater passive release of exudates. Soil nitrogen has a similar relationship to rhizodeposition. Although high concentrations of available nitrogen tend encourage above-ground plant growth at the expense of roots, N availability increases root branching, thus increasing rhizodeposition in relation to total below-ground C (Nguyen 2003). This relationship may, however, depend on plant species. Van der Krift et al. (2001), found that root biomass production and rhizodeposition were positively correlated with N availability only for species adapted to high soil N concentrations.

Rhizodeposit quality, defined by N concentration in rhizodeposits, is primarily a function of total assimilation and concentrations of nitrogen by plants. Due to their capacity to assimilate

atmospheric N_2, legume rhizodeposits have lower C/N ratios than cereals (Cheng et al., 2003; Dijkstra et al., 2009; Wichern et al., 2007; Zhu & Cheng, 2012). Soil conditions also affect rhizodeposition of N, insofar as they regulate plant N uptake. In two studies, wheat grown in low N conditions lost less N through its roots as a percentage of its total biomass N than wheat grown in high fertility soil (Janzen & Bruinsma, 1989; Janzen, 1990). Nitrogen lost as rhizodeposits had linear relationship to total plant N (Janzen and Bruinsma, 1989). Legumes, on the other hand, may behave differently than non N_2-fixing plant species. Arcand et al. (2013) found that fertilized pea assimilated more total N, yet did not release significantly more rhizodeposit N as a percent of total plant N than non-fertilized pea.

Rhizodeposition as a source of nitrogen has been understudied, yet recent research suggests that significant amounts of plant N are released into the soil during plant growth as rhizodeposits, especially by legumes. However, both legumes and cereals exhibit similar ranges of N allocated below-ground as a proportion of total plant N, with of approximately 35% (Wichern et al., 2008).

In a review by Wichern et al. (2008), results from studies measuring N rhizodeposition were tabulated and interpreted in terms of NdfR as a % of BGN, NdfR as % total plant N, and BGN as % total N. Their conclusions are worth summarizing. Estimates of NdfR in peas range from 4%-71.1% of total plant N, (mean: 22%) representing 15%- 96% (mean: 68%) of BGN. However, methods and growth conditions vary, and these means must be scritinized. Using the wick method, NdfR ranged from 13% (Mayer et al. 2003) to 18% (Arcand et al. 2013) of total plant N. However, using larger soil volumes, Wichern et al. (2007b) reported that NdfR was 29% of total plant N, which corresponds to increased root growth. On the other end, plant growth stage influenced amounts of rhizodeposited N. At early vegetative growth, NdfR accounted for 71% of total plant N, yet averaged throughout growth period, NdfR equaled 29% of total biomass N. A more conservative mean considering only studies deploying the wick method, then, suggests a slightly higher mean of NdfR as 27% of total plant N. In all these of studies, NdfR represents 76-87% of BGN, suggesting that peas an extremely high percentage of fixed-N from roots. Other legume crops, grown under similar experimental conditions, fall within this range, with NdfR ranging from 9- 44% of total plant N with a mean of 21%. However, NdfR in % of BGN ranged from 24-88, indicating important species differences amongst legumes. Estimates of NdfR in cereals also ranges widely, although there are, unfortunately, fewer methodological consistencies amongst studies. In studies of wheat, barley and oats, NdfR ranges from 4.3-56% of total plant N with a mean of 17%, representing an average of 57% of BGN.

Few studies have investigated N rhizodeposition in intercrops, but evidence suggests that incropping can increase N rhizodeposition. It is difficult compare quantities of rhizodeposition from *in situ,* multi-year studies to single-year greenhouse studies. Recovered soil ^{15}N will reflect not just rhizodeposition, but the net results of multiple processes including N- transfer, root senescence, decomposition of root residues and uptake of rhizodeposited N. In a two-year study of N rhizodeposition in clover-ryegrass pasture, red clover-grass mixes released 89 g N m^{-2}, compared to 64 g N m^{-2} in red-clover monocultures. However, in this study, mixed swards accumulated less root biomass, and 25-50% less N than pure red and white clover stands respectively. Due to the duration and methods of this study, NdfR as a percentage of total plant N cannot be properly determined (Hogh-Jensen and Schjoerring, 2001). Rasmussen et al. (2008), in another clover-ryegrass study examining N transfer as well as N rhizodeposition, found that NdfR represented 36% of total plant N (both clover and ryegrass), and 71% of BGN, comfortably within the ranges of the studies reported by Wichern et al. (2008). In addition, Rasmussen et al., (2008) reported that 40% of ryegrass N originated from associated clover, suggesting that total N rhizodeposition was likely much higher than indicated by residual soil N.

1.4 Decomposition, stabilization and mineralization of root-derived nitrogen

Recent developments have significantly transformed our understanding of soil organic matter (SOM) dynamics, especially processes linking residue decomposition, microbial uptake, and formation of labile SOM. New research emphasizes the relative significance of chemical adsorption over physical protection or recalcitrance in organo-mineral associations (Marschner et al., 2008; Schmidt et al., 2011; Sollins et al., 2009) and predominance of microbial byproducts in SOM (Kallenbach et al., 2015; Ludwig et al., 2015;Cotrufo et al. 2013; Kindler et al., 2009; Miltner et al., 2012). In particular, the microbial efficiency—matrix stabilization (MEMS) framework (Cotrufo et al., 2013; Castellano et al., 2015) links stoichiometry of plant residues to SOM stabilization through mechanistic understandings of microbial metabolism and electro-chemical binding. MEMS posits that SOM forms when microbial products of decomposition are stabilized via chemical bonding to the soil mineral matrix. Observing that labile, low C/N plant residues are more efficiently metabolized and converted to microbial biomass relative to high C/N plant material, MEMS suggests that plant residues with low C/N (high N content) are preferentially stabilized is the primary source of SOM. As the N transformation is tightly coupled to C cycling in soils, these developments have been equally insightful in terms of understanding N transformations as SOM formation (Bingham & Cotrufo, 2015).

In terms of C, root residues tend to decompose more slowly than shoot residues, and are known to contribute significantly to the buildup of soil organic matter pools (Gale et al., 2000; Puget & Drinkwater, 2001; Rasse et al., 2005; Williams et al., 2006). Root chemistry is important for explaining decomposition. In non-N limiting conditions, residue decomposition is primarily correlated with concentrations of soluble C components (Trinsoutrot et al. 2000). Concentrations of recalcitrant components such as lignin, cellulose and hemicellulose content will limit decomposition once labile compounds are released, and can contribute to slower decomposition of roots. (Abiven et al., 2005; Müller et al., 1988; Silver & Miya, 2001). Fine roots, with smaller diameter and lower concentrations of lignin, tend to decompose more rapidly than coarse roots (Jani et al., 2015). Roots of differing diameter may also contribute differently to soil organic matter pools. Rasmussen et al., (2010) found that small roots of clover and rye-grass contribute to buildup of soil N, while coarse roots are the main source of organic C accumulation. Plant age can also affect chemical characteristics of roots. Older roots tend to have greater lignin concentrations and high C/N ratios than younger roots, as N is translocated from roots to shoots during reproductive growth (Gardener and Sarrantonio, 2012).

Net N mineralization associated with root decomposition can be predicted largely by C/N ratios of residues (Abiven et al., 2005; Bolger et al., 2003; Schomberg et al., 2005; Silver & Miya, 2001; Trinsoutrot et al., 2000). N mineralization tends to occur when N is abundant relative to C, and C is limiting for microbial growth. Microbes target SOM for C, and release N as NH_4^+ into soil solution. In contrast, N limitations in the presence of labile C can stimulate SOM decomposition and CO_2 mineralization, as microbes mine substrate for N content (Carrillo e al., 2014; Craine et al., 2007). C/N ratios above 20-25 tend to induce immobilization of N in microbial biomass (Silver & Miya, 2001; Trinsoutrot et al., 2000), although mineralization has been reported at C/N values as high as 37 (Chaves et al., 2004). The turnover and decomposition of fine roots, with their lower C/N ratios and high concentration of soluble compounds, tend to result in net N mineralization (Bolger et al., 2003; Fornara et al., 2009). Residues with wide C/N values often show initial period of immobilization, followed by a period of N mineralization (Chaves et al., 2004; Parr et al., 2014). Net N mineralization, however, can be decoupled from residue decomposition. Soil mineral N may be immobilized without decreasing the rate of decomposition or increasing microbial biomass, as rapid turnover of microbial biomass can sequester N microbial residues and stable organic matter pools (De Neergaard et al., 2002). In a study of root and rhizodeposit decomposition, Mayer et al., (2004) found that only 12-32% of mineralized N was derived from rhizodeposition; a majority of rhizodeposited N was found in either microbial residues or undecomposed fine roots. Malpassi et al., (2000) reported, over the

course of a 112 day incubation, oat and rye roots lost ~3 micrograms N g soil $^{-1}$, yet soil mineral N increased by three times that amount, indicating that a majority of inorganic N was derived from other labile pools of soil organic N.

Despite the slower decomposition of root residues compared to aboveground biomass, more recent work has confirmed the preferential stabilization of root C in stable fractions of soil organic matter. Mambelli et al., (2011) report a selective retention of root-derived C and N in particulate organic matter fractions compared to needles in forest soils, and Mazzilli et al. (2015) found that 60 to 80% of particulate organic matter in agricultural soils was derived from belowground biomass after two growing seasons. The discrepancy between delayed mass-loss decomposition and accumulation of root C and N in stable SOM pools is difficult to parse. It may be that while roots contain higher concentrations of structural C components, root N is more readily available for metabolism by soil microbes. Uselman et al. (2012) report accumulation of soluble, labile N with increasing root inputs in forest soils. In a three-year decomposition, Sanaullah et al. (2011) reported rapid assimilation of root N in microbial biomass within six months, followed by a steady decrease in percent root N in microbial biomass. Similarly, they observed a decrease in root-derived N in labile fractions of SOM, and an increase in root-N accumulating in the aggregate-occluded and mineral-associated fraction.

The persistence of root-derived N in has been demonstrated in many studies. In one 3-year pea-residue decomposition study, only 2-3% of remaining residual N was potentially mineralizable, indicating that a majority of residue N was stabilized in various SOM pools (E.S. Jensen, 1994). Revisiting this study 8 years later, Laberge et al., (2006) found that 24% of pea residue remained in soil. After 16 years, 16% of residue N remained. Glasener et al. (2002) confirmed that a majority of N derived from root residues remained in SOM pools over three cropping events, while mineral N from residues recovered in soil decreased after each harvest.

Due to the persistence of crop residues in SOM pools, cropping system dynamics such as tillage (Panettieri et al., 2014), cropping frequency (Gosling et al., 2013), and quantity of organic C inputs (Spargo et al., 2011; St. Luce, 2016) can all can have significant effects on quantities and qualities of SOM-N pools (Bu et al., 2015). Residue quality is important for SOM-N transformations in the short term. In a 120 day incubation, 24-25% wheat residue-N accumulation in labile organic matter fraction, compared to 14-16% of fava bean residue, while N mineralization was positively correlated to initial concentration of N in labile fractions (St. Luce et al., 2014). On a longer time scale impacts of residue quality on SOM-N pools are likely small compared to other management factors (Chivenge et al., 2011; Fonte et al., 2009). Thus, despite overwhelming evidence for short-term SOM-N stabilization of residue decompositions, field

scale-differences in SOM-N often become apparent only after several years (Panettieri et al., 2014). Cropping frequency and C inputs were found to be a major factor determining quantity of particulate organic matter in agricultural fields. In a meta-analysis of 150 experiments, Gosling et al. (2013) found that the presence of fallow for 20, 25, and 30% of a rotation decreased particulate organic matter accumulation by 20, 24, and 30%, while organic amendments such as manure increased accumulation of labile SOM by 30-40%. Spargo et al. (2011) reported a significant increase in potentially mineralizable N in organic plots due to history of manure application, 235 kg ha^{-1} in conventional to 315 kg ha^{-1} in organics. Perennial cover with alfalfa can increase labile SOM concentrations (Spargo et al., 2011), and the proportion of soil N contained labile SOM. St. Luce et al, (2016) found that labile SOM contained 28% of total SOM-N under alfalfa, compared to 9 and 11% under corn-corn and corn-soy-forage rotations.

Fewer studies have isolated the effects below-ground residues on SOM-N accumulation in soils, although evidence points towards the importance in C input quantity on root-derived N stabilization. Winter cover crop residue removal over four years was found to decrease soil organic C and N, and decrease C:N ratios (Kuo & Jellum, 2002). In a three year study with residue removal and N addition, Fonte et al., (2009) found that N fertilization decreased aggregate stability regardless of residue input quality. These results are consistent with findings that while root-derived N accumulates in stable SOM fractions, C limitations can result in decomposition of native SOM, rather than accumulation of new SOM.

1.5 Availability of root-derived nitrogen to subsequent crops

Accumulated labile SOM-N pools is an important source of N, as a majority of N mineralized from soil organic matter is derived from the labile pools (St. Luce et al., 2014; Marriott & Wander, 2006). Living roots can access SOM-N through the rhizosphere priming effect (Kuzyakov, 2002). Rhizodeposition of labile C compounds can stimulate decomposition of native soil organic matter, increasing soil inorganic N concentrations (De Graaff et al., 2007; Schweinsberg-Mickan et al., 2012; Zhu et al., 2014). St. Luce et al, (2016) found that the addition of labile SOM with high N concentrations resulted in greater N mineralization than addition of low-N SOM. Both recently incorporated residues and accumulated SOM-N can be significant sources of N for crops. Subsequent crops typically take up 8-17% of BGN in the first year after residue incorporation (Arcand et al., 2014; Laberge et al., 2011; Mayer et al., 2003; Russell & Fillery, 1996). The proportion of total plant N that this uptake represents also varies considerably. In the first year after lupin residue incorporation, 40% of N in wheat shoots was derived from residues in unfertilized treatments, while residue N only accounting for 15-20% in fertilized

treatments. In the second year, however, both 15% of N in wheat came from residue N (Thompsen and Fillery, 2002). In another study, red clover residues provided subsequent corn with an estimated 20-36% of corn N uptake (Gentry et al., 2013). Accumulated soil N can also meet N demand. Spargo et al. (2007) report organic plots, with history of manure inputs took up 195 kg N ha^{-1} compared to 132 in conventional, and yielded 9.61 Mg grain ha^{-1}, average for organic corn without additional fertilizer N.

1.6 Conclusion

Below-ground residue contributions to subsequent crops occurs at two time frames. In the short term, BGN is immobilized into microbial biomass, or mineralized to NH_4^+-N through decomposition. In the medium- and long- terms, residue N cycles through microbial biomass and accumulates as microbial residues in labile SOM fractions, or sequestered as occluded or mineral-associated SOM-N. The former is then subject to mineralization. Several gaps exist in understanding the contributions of BGN to subsequent crops. The quantities and properties of root and rhizodeposit N are the primary determinants of SOM-N stabilization. While factors such as root density root age and species diversity and function, are well documented, rhizodeposit N is still, in many regards, a mystery. It is posited that rhizodeposit N is primarily due to fine-root turnover and root death rather than amino acid exudates, which means that rhizodeposition N is likely to function similarly to high quality, labile N inputs. However, measurements of quantities of rhizodeposition N need refinement. Another gap is the discrepancy between root chemical properties and N decomposition. While roots tend to have higher C/N ratios, higher concentrations of structural C compounds, and exhibit slower decomposition (Abiven et al., 2005; Gardner & Sarrantonio, 2012), they are also preferentially sequestered into stable SOM fractions compared to above-ground residues (Mambelli et al., 2011; Mazzilli et al., 2015; Sanaullah et al., 2011). A final gap concerns factors affecting accumulation of SOM-N over longer time frames. Longer term field involving above-ground residue removal show decreased accumulation of SOM-N (Fonte et al., 2009; Kuo & Jellum, 2002; St. Luce et al., 2016). Given that management practices such as tillage, and quantity of biomass C inputs seem to be significant for SOM-N development, it may be that the capacity long-term accumulation of root-derived SOM-N is limited by residue removal in high-intensity cropping systems.

2 Double-cropping legume-based forage and early-maturing corn as a low N-input cropping system in Minnesota

2.1 Abstract

Double-cropping with forages can increase yields and N-use efficiency over sole-crop systems, but reductions in primary crop yield can limit economic returns. This study assessed whether the combination of high value, forage, early maturing corn varieties, and reduced N inputs constitutes an economically viable, low N-input double crop system for Minnesota. Biomass yield, N uptake, and residual soil N were measured in two double-crop (DC) and one sole-crop (SC) systems at site-years in MN, from 2014 to 2016. In DC treatments, a pea- (*Pisum sativum L.*) barley (*Hordeum vulgare L.*) forage bi-culture was double-cropped with early-maturing hybrid (DC-HC) or semidwarf (DC-SD) corn (*Zea mays*, L.) varieties. In SC treatments, full-season hybrid corn (SC-HC) was planted with no preceding forage. Corn was supplied with 6 N rates (0 to 224 kg N ha^{-1} for each yield component. Corn yielded less biomass in DC-HC (8.2 Mg ha^{-1}) and DC-SD (1.8 Mg ha^{-1}) treatments yielded compared to SC-HC (16.3 Mg ha^{-1}). Biomass yield deficits lowered corn N demand in DC-HC treatments so that N rates >166 kg N ha^{-1} did not limit biomass yield in DC-HC treatments, where SC-HC corn was limited by N rate in three of four site-years. Total biomass accumulation was similar between DC-HC and SC-HC treatments when forage bi-culture yielded >7 Mg ha^{-1}. This suggests that double-cropping with high-quality forages may constitute an economically viable low N-input alternative to sole-crop corn production in Minnesota.

2.2 Introduction

In the U.S. corn-belt, the industrialization of agriculture since World War II, and the decoupling of crop and livestock production, has led to a simplification of agricultural land-use, with continuous, annual corn- (*Zea mays L.*) soy (*Glycine max L.*) cropping systems replacing long-term rotations and perennial pasture (Sulc & Tracy, 2005). A host of deleterious ecological consequences have accompanied this changes, including widespread soil C losses (Lal, 2004; Moran & Jastrow, 2010; Olson, 2013), increased nitrate pollution in waterways due to nitrogen (N) leaching and surface runoff (Bierman et al., 2012; Strock et al., 2004), and increased greenhouse gas emissions (McSwiney et al., 2010; Stavi & Lal, 2013). The re-integration of crop and livestock systems may be an opportunity to mitigate the impacts of industrialized agricultural production through crop rotations, tightly coupled N cycling, and risk management using systems diversification (Russelle et al., 2007). As the broader socio-economic dynamics that drive the

trend towards specialization are unlikely to reverse course, recent agronomic systems research and innovation at the University of Minnesota has focused on diversifying agricultural production without disrupting predominant land use patterns (Jordan & Wyse, 2015).

Double-cropping, which can increase yields and N-use efficiency over sole-crop systems, is one strategy to incorporate forages into grain production systems. In double crop systems, two crops are harvested from the same field in a single season. Commonly, a winter cereal crop is harvested as forage in the spring and followed directly by a warm-season, primary crop. Double cropping with winter forage can provide many of the benefits associated with winter annual cover cropping, such as the retention of soil organic N and reduced N leaching (Ketterings et al., 2015; Krueger et al., 2011), and increased soil organic matter (Kuo & Jellum, 2002), in addition to the production of high quality forage (Brown, 2006; Jemison et al., 2012). Biomass accumulation in double-crop systems is positively correlated with leaf area duration (Heggenstaller et al., 2009). Double-crop systems can increase leaf area duration in two ways. First, by expanding growing period to include late fall and/or early spring, which allows for increased utilization of soil resources such as N. Second, cool-season crops with lower base growth temperature requirements than warm-season crops produce biomass more efficiently at cool temperatures, and accumulate more growing degree units (GDU) in spring and fall (Mirsky et al., 2009; Teasdale et al., 2004). Biomass yield in double-crop systems can be 25% greater than sole-crop systems (Heggenstaller et al., 2008; Jemison et al., 2012). However, a reduced growing season and competition between spring forage and primary crop for nutrient and moisture resources (Krueger et al., 2010; Krueger et al., 2011) can also reduce primary crop yields compared those in sole-crop systems (Heggenstaller et al., 2008; Jemison et al., 2012; Krueger et al., 2012; Thelen & Leep, 2002). For double cropping to be economically viable in annual corn-soybean systems, corn yield must be maintained, and the returns from the spring forage must compensate for the costs of additional field operations (Borchers et al., 2014). Due to Minnesota's short growing season, a double-crop system that prioritizes economic value rather than season extension, may result in a cropping system that meets these requirements. Further, as no annual forage legume are winter hardy in Minnesota, the forage crop is restricted to spring planting instead of fall, which can delay corn planting.

Spatial intercropping legumes with cereals, also known as bi-cultures, results in greater overall N uptake and higher forage quality than mono-cropped cereals (Carr et al., 1998; Chapagain & Riseman, 2014; Lauriault & Kirksey, 2004). Pea (*Pisum sativum L.*) and barley (*Hordeum vulgare L.*) bi-cultures have been shown to yield up to 13.5 Mg ha^{-1} DM biomass, with crude protein content (CP) of 12.7% (Strydhorst et al., 2008). Selection of early-maturing primary

crop varieties can compensate for a shortened growing season associated with spring-planted forages (Jemison et al., 2012; Kyei-Boahen & Zhang, 2006). While double crop systems typically utilize silage corn or warm season forage grasses as primary crops, some studies have successfully demonstrated the suitability of early maturing grain corn varieties for these systems (Heggenstaller et al., 2008; Thelen & Leep, 2002). Semidwarf corn with 62-day relative maturity (RM) was reported to yield 5 Mg grain ha^{-1} in Minnesota, and its rapid maturation may fit well into double-crop systems (Schaefer et al., 2011).

Additionally, the inclusion of legumes in a double-crop system may allow for nitrogen (N) fertilizer input reduction, potentially offsetting forage production costs. It is well established that decomposition of legume biomass can increase N availability in soil (Redin et al., 2014), and that cereal-legume bi-cultures can be managed to provide N to subsequent crops (Cook et al., 2010; Glasener et al., 2002; Kuo & Sainju, 1998; Lawson et al., 2012; Tonitto et al., 2006). Decomposition of below-ground cover crop root residues can result in N mineralization, even if above-ground biomass is removed as forage (Kuo & Jellum, 2002; Malpassi et al., 2000). Fewer studies have isolated the contributions of below-ground residues to subsequent crop N uptake. Kuo and Jellum (2002) reported a reduction in soil N following removal of cereal-legume bi-culture compared to cereal mono-culture. However, Yost et al. (2014) found that corn shows no response to applied N fertilizer for two years following alfalfa (*Medicago sativa L.*) termination, allowing for a potential reduction of about 168 kg N ha^{-1} (Yost et al. 2012). As alfalfa is a perennial forage crop managed with continuous biomass removal, this suggests a strong potential for N credits from legume root decomposition. Thus, there may be potential to manage annual, cereal-legume forage crops as a source of N for subsequent crops.

The objective of this study was to assess whether the combination of high value, cereal-legume forage with early maturing corn varieties, and reduced N fertilizer inputs constitutes a viable, low N-input double crop system that integrates forage and grain production in Minnesota. I hypothesized that 1) spring annual bi-cultures can economically yield high-value forage in a double-crop system, 2) early maturing corn varieties will yield grain in a double-crop system, 3) bi-culture forage—corn double crop systems have require less N fertilizer to optimize economic returns than sole-crop grain corn.

2.3 Materials and methods

2.3.1 Sites and experimental design

Two double-crop (DC) and one sole-crop (SC) systems supplied with six N rates (0 to 224 kg N ha^{-1}), followed by a winter rye cover crop, were studied from 2014 to 2016 at three

locations at Minnesota. The study was conducted at the Southwest Research and Outreach Center (LAM) in Lamberton, MN (44.0707°, -93.5264°) on a Normania loam (Fine-loamy, mixed, superactive, mesic Aquic Hapludol) and the Rosemount Research and Outreach Center (ROS) in Rosemount, MN (44.7178°, -93.0975°) on a Waukegan silt loam (Fine-silty over sandy or sandy-skeletal, mixed, superactive, mesic Typic Hapludol) from May 2014 through June 2015, and continued at LAM and the Southern Research and Outreach Center (WAS) in Waseca, MN (44.0707°, -93.5264°) on a Glencloe clay loam (Fine-loamy, mixed, superactive, mesic Cumulic Endoaquoll) from April 2015 through April 2016 under rain-fed conditions.

The experiment design was a randomized complete block design in a split-plot arrangement, with three crop treatments as the main-plot treatments and six N rates the sub-plots, with four blocks at each site. Plot size was 3 by 14.6 m at ROS and WAS, and 3 by 10 m at LAM. In DC treatments, a bi-culture of spring planted pea (variety 4010) and barley (variety Morex) was double-cropped with early-maturing hybrid corn (HC) or semidwarf (SD) corn varieties. A dual-purpose, silage-grain corn (variety Pioneer 8906 AM1; RM=89) was used in DC-HC treatments, and semidwarf corn variety COPOP1 (Schaefer et al., 2011) (RM=62) in DC-SD treatments. In SC treatments (SC-HC), full-season, glyphosate-tolerant hybrid corn (variety Pioneer 0297 AMX; RM=102) was planted with no preceding forage. Six N fertilization rates (N0, N45, N90, N135, N179, and N224) were applied as broadcast urea at corn planting in all crop treatments. Cereal rye (*Secale cereale* L. 'Rymin') was planted in all treatments following corn harvest in plots, and harvested as forage at boot stage, i.e. Zadoks 40 (Tottman, 1987) the following spring (Table 2.1).

2.3.2 Agronomic management

Pea-barley bi-culture was planted in DC treatments on 5 May in 2014 and 2 April at WAS and 6 April at LAM in 2015 (Table 2.1), at rates of 96 (barley) and 67 (pea) kg live seed ha^{-1} with a grain drill (Model 8300, John Deere, Deere and Company, Moline, IL) at 18 cm row-spacing. Sole-crop corn was seeded at a rate of 79,090 live seed ha^{-1} on 6 May in 2014, and 1 May (LAM) and 17 April (WAS) in 2015 (Table 2.1), using John Deere 7000 MaxEmerge planter at 76 cm row-spacing. Following spring forage harvest, semidwarf corn was seeded on 3 July in 2014 and 2 July (WAS) and 15 July (LAM) in 2015, at 50 cm row-spacing at seeding rates of 395,452 using a cone drill. Double-crop hybrid corn was seeded at 76 cm row spacing at 79,090 live seed ha^{-1} using a John Deere 8300 drill. All plots were treated with of 0.8 kg a.i. ha^{-1} S-metolachlor pre-corn emergence.

Pea-barley forage was sampled in June when barley was at Zadoks 85 (soft-dough stage) in 2014 and Zadoks 90 in 2015. Forage biomass samples were taken block-wide from 0.25 m^2 quadrats. Pea and barley biomass was separated, weighed and dried at 35 °C to determine dry matter content. After forage harvest, the forage plots were disked and prepared for corn planting. Glyphosate (*N*-(phosphonomethyl)glycine) was applied to corn in hybrid corn treatments at rate of 1.1 kg a.i. ha^{-1}, and semidwarf corn was treated with tembotrione (2-[2-chloro-4-(methylsulfonyl)-3-[(2,2,2-trifluoroethoxy)methyl]benzoyl]-1,3-cyclohexanedione) at 29 g a.i. ha^{-1} to control weeds. Semidwarf corn was not glyphosate resistant, so Laudis was selected for post-emergence weed control.

All crops were sampled for above ground biomass, and either grain or silage yield in October (Table 2.1). Grain and silage yield was collected from 6 m length within plots in corn treatments; stover biomass was collected from a 3 m length within plots. After removal of all corn residue, the field was lightly disked.

Following corn, cereal rye was planted at 18 cm row-spacing using a John Deere 8300 grain drill at a rate of 134 kg live seed ha^{-1}. Rye was harvested from all plots at inflorescence (Zadoks 59) using 0.56 m^2 quadrats, dried at 60°C and weighed for DM biomass. Although systems were managed across two calendar years, year of initial system components will be used to describe all components of system; thus, rye planted in fall 2014 and harvested in spring 2015 will be is considered as occurring in '2014.'

2.3.3 Climate and growing degree units

Climate data was collected from weather station data, accessed from Midwest Regional Climate Center (MRCC). Growing degree units were calculated as:

$$GDU = \frac{(T_{max} - T_{min})}{2} - T_{base}$$

where if $T_{max} < T_{base}$, then $T_{max} = T_{base}$, and if $T_{min} < T_{base}$, then $T_{min} = T_{base}$.

T_{max} is maximum reported temperature, T_{min} is minimum reported temperature, and T_{base} is minimum growing temperature required by crop (McMaster & Wilhelm, 1997). T_{base} for corn was set at 10 °C, and T_{base} for pea, barley and cereal rye was 4 °C (Mirsky et al., 2009; Teasdale et al., 2004)

2.3.4 Soil

Soils samples were collected to a depth of 30 cm at three times throughout the study: prior to corn planting (base), at corn harvest (fall), and at rye harvest (spring). Base samples were aggregated from all plots, prior to corn planting and N application, as crop and N rate treatments had not yet been assigned. For fall and spring samples, six cores per plot were aggregated and subsampled. All samples were dried at 35 °C and ground to <0.5 mm. Soil was analyzed for NO_3-N and NH_4-N by Agvise Laboratories (Benson, MN).

Nitrogen balance was calculated as:

$$N_{balance} = (N_{base} + N_{rate}) - N_{corn}$$

Where N_{base} is base soil N, N_{rate} is N applied as fertilizer in Nrate treatments, and N_{corn} is corn N uptake at harvest.

2.3.5 Sample analysis

All biomass samples were dried at 60°C, weighed for dry matter (DM) and ground to pass through a 6 mm screen using a Thomas Wiley mill (Thomas Scientific, Swedesboro, NJ). Coarse ground samples were mixed, subsampled and ground to pass through a 1 mm screen using a Cyclotec Sample Mill (FOSS North America). Total DM was calculated as the sum of forage bi-culture, corn and rye DM. All biomass components were analyzed for crude protein (CP). Biomass N content was calculated for all samples as: N% DM = CP/6.25. Corn silage and forage bi-culture, was analyzed for forage nutritive parameters: CP, acid detergent fiber (ADF), neutral detergent fiber (NDF) and 48-hour in vitro NDF digestibility (NDFD), using near infrared reflectance spectrometry (Perten, DA 7250). Relative forage quality (RFQ) was calculated for forage-bi culture using equations described by Jeranyama & Garcia (2004).

2.3.6 Economic analysis

Net economic returns for each treatment were calculated as the difference between production costs and gross value per hectare. Fertilizer prices were 2014 prices for Minnesota from the National Agricultural Statistics Service (USDA-NASS, 2015), and herbicide prices were 2014 prices from the North Dakota State University Extension (Zollinger et al., 2014). Field operation costs were estimates from University of Minnesota Extension (Lazarus, 2014). Labor, repairs, fuel, depreciation and machinery overhead, which included interest, insurance and housing, comprised total cost of field operations. Crop prices, based on average of 2010-2014

prices received by Minnesota producers, were as follows: Corn grain: \$0.20 kg^{-1} (USDA-NASS, 2015), Corn silage: \$37.35 Mg^{-1} (FINBIN, 2015), pea-barley forage: \$136.52 Mg^{-1} (FINBIN, 2015) and rye forage: \$70.17 Mg^{-1} (FINBIN, 2015).

Grain yield was calculated as grain dry matter yield at 15.5% moisture; silage yield was calculated as corn silage dry matter yield at 65% moisture, and rye and pea-barley forage yield was calculated as dry matter yield at 15% moisture.

2.3.7 Statistical analysis and models

Due to strong interaction effects based in environment, all analysis was conducted separately by site-year environment. Analysis of variance (ANOVA) was conducted for DM yield, N uptake, fall soil N, forage nutritive parameters, and calculated economic returns, through PROC MIXED in SAS/STAT (SAS Institute, 2004). Crop and N rate treatments were considered fixed effects, and blocks and crops within blocks were considered random effects. Where necessary, data was e^x transformed to meet ANOVA assumptions. Least square means of fixed effects were compared at $P = 0.05$ using Tukey's HSD performed by a SAS pdmix800 macro (Saxton, 1998).

Quadratic or quadratic plus plateau models were selected to describe corn dry matter yield and N uptake response to N rate (Cerrato and Blackmer, 1990). The equation for quadratic plus plateau model was:

$$Y = aX^2 + bX + c, \text{ if } X < X_{max}, \text{ and}$$
$$Y = X_{max} \text{ if } X \geq X_{max}, \text{and}$$

Quadratic model was:

$$Y = aX^2 + bX + c,$$

Where Y was DM yield N uptake (kg ha^{-1}), X is N rate, a and b are quadratic and linear coefficients, c is intercept. X_{max} is the optimum N rate (ONR) that occurs at intersection of two functions in quadratic plus plateau model.

2.4 Results

2.4.1 Climate and GDU

Lamberton received lower average seasonal precipitation (663 mm, Jan-Oct) compared to ROS 2014 (842 mm) and WAS 2015 (955 mm) (Figure 2.1). In 2014, heavy precipitation in April delayed double-crop (DC) forage planting until sole-crop (SC) planting in May at both sites (Table 2.1). In July 2014, LAM and ROS experienced average rainfall deficits of 50% and 70%, respectively, with deficit extending through September at ROS (Figure 2.1). Waseca in 2015 had favorable spring conditions, allowing early establishment of DC forage and SC-HC treatments compared to 2014 environments (Table 2.1). However, rainfall and flooding following forage harvest at WAS delayed field preparation for DC corn until mid-July. Subsequent flooding at this location resulted in the forfeiture of data from all DC-SD treatments within one block, and half of DC-HC treatments within another.

Deviation in temperature from historical means occurred primarily between October and April (Figure 2.1). Thus, planting dates and climatic differences across locations were responsible for differences in growing degree unit (GDU) accumulation across crop treatments (Table 2.2). In 2014, DC forage accumulated 740 GDU in LAM and 783 in ROS, though growing days were the same at each location (Figure 2.2). For DC forage in 2015, both growing days and GDU accumulation were greater at WAS. On average across ROS 2014 and LAM 2014 and 2015, SC-HC accumulated 53% more GDU than either DC treatments. In WAS 2015, delayed DC corn planting led to 75% GDU deficit in DC treatments compared to SC treatments (Table 2.2). However, the sum of forage and main crop GDU in DC treatment results in a 20% GDU advantage for DC treatments in ROS 2014 and LAM in both years, and a 12% GDU advantage in WAS 2015.

2.4.2 Biomass yield and N uptake

Double crop forage dry matter (DM) yielded between 2.9 and 13.3 Mg ha^{-1} (Table 2.3). Forage DM yield was positively correlated with GDU, ($P < 0.001$, $R^2 = 0.93$), increasing 0.085 Mg DM GDU^{-1}. Pea comprised 16 to 37$ of forage DM, and N uptake ranging from 53 and 252 kg N ha^{-1} (Table 2.3). Due to favorable conditions at WAS 2015, barley reached grain ripening stage (Zadoks 90; Tottman, 1987) prior to harvest. Nutritive parameters for forage bi-culture were similar across environments. Mean CP, ADF, NDF concentrations of 107, 317, and 522 g kg^{-1}, respectively (Table 2.3), where similar to values of 124, 267, and 432 g kg^{-1} reported for pea-barley bi-culture by Strydhorst et al. (2008).

Corn DM yield varied significantly by crop treatment in all environments, N rate at ROS 2014 and WAS 2015, and by treatment interactions at all environments except LAM 2015 (Table 2.4). Dry matter yields consistently ranked DC-SD < DC-SC < SC-HC. Dry matter yield in DC-DS treatments where not greater than 2.5 Mg ha^{-1}, and DC-HC yields were, on average, 51% lower than SC-HC yields (Table 2.4). However, in 2014 treatments supplied with lower rates of N (N0, N45, N134 in LAM2014; N0-N90 in ROS 2014), DM in DC-HC and SC-HC treatments were not significantly different. This trend was not observed at LAM 2015, where no N rate effect was seen, standard error in DM yield within N rate treatments was 22%.

Nitrogen uptake by corn was primarily a function of DM yield. Biomass N averaged 1.6% of DM yield in DC-SD and 1 and 1.2% of DM yield in DC-HC and SC-HC treatments, respectively, with little variation across N rates (Table 2.5). Nitrogen uptake was strongly affected by crop and N rate treatments, with interaction effects at ROS 2014 and LAM 2015.

Corn DM yield in DC-SD treatments was unaffected by N rate at all locations except LAM 2014 (Table 2.4). There, no clear trends were apparent, with peak DM yield (2.3 Mg ha^{-1}), and minimum yield (1.2 Mg ha^{-1}) occurring in N0 and N179 treatments. Semidwarf corn reached physiological maturity, except at WAS 2015, where grain production was agronomically negligible and thus not separated from stover during harvest. Grain yield averaged 0.79 Mg ha^{-1}, or 40% of SD corn biomass (data not shown). Although DC-SD had greater biomass N concentration than hybrid corn, N uptake by SD corn was limited by DM yield, and did not surpass 47 kg N ha^{-1}.

Double-crop hybrid corn did not reach physiological maturity, and was harvested at soft dough stage for silage. Dry matter yield varied considerably across sites, ranging from 5.8 Mg ha^{-1} to 12.4 Mg ha^{-1}, and a mean of 8.2 Mg ha^{-1} (Table 2.4). Maximum DM yields were 10.4, 10.1, 8.6 Mg ha^{-1} at ROS 2014, LAM 2015 and WAS 2015, respectively. Nitrogen uptake by DC-HC increased with N rate, and trend was significant at all locations except LAM 2014 (Table 2.4).

Dry matter yield and N uptake responses to N rate by corn in DC-HC and SC-HC treatments were fit with quadratic plateau models (Figures 2.3 and 2.4). All models were significant fits, except for DM response at LAM 2014 (Figure 2.2a). For significantly fitted models, predicted ONR for DM and N uptake by DC-HC treatments was not greater than 166 kg N ha^{-1}, within range of rates provided in this study (Figures 2.3 and 2.4). Predicted ONR for N uptake by DC-HC was similar to those for DM yield, except at WAS 2015, where ONR for N uptake was 90, compared to 136 kg N ha^{-1} for DM yield (Figure 2.4d). In contrast, predicted ONR were greater than 224 kg N ha^{-1} for DM yield in ROS 2014 and WAS 2015 (Figure 2.3a,d), and

for N uptake at WAS 2015. No ONR was calculated for N uptake by SC-HC at ROS 2014, which was fit to a quadratic model without plateau.

Rye DM yield averaged from 1.6 Mg ha^{-1} at WAS 2015 in SC-HC treatments to 4.7 Mg ha^{-1} in DC-SD treatments at LAM 2015 (Table 2.4). In 2014, rye DM yield was not affected by crop, N rate, or treatment interactions. In 2015, rye DM yield was significantly greater in DC-SD than DC-HC or SC-HC treatments, but only increased with N rate in DC-SD treatments at LAM 2015. Rye N uptake increased with N rate at all environments, and was greatest in DC-SD treatments (Table 2.5).

Total system DM was significantly affected by crop and N rate at all environments, with significant treatments interactions in all sites except LAM 2015 (Table 2.4). Except at LAM 2014, total DM in DC-HC treatments where similar to SC-HC treatments averaged across N rates due to the presence of forage bi-culture in the DC systems (Table 2.4). Forage contributed 26, 30, 41, and 60% of total DC-HC DM in LAM 2014, ROS 2014, LAM 2015 and WAS 2015, respectively. In N0 treatments at ROS 2014 total DM in DC-HC was 34% greater than in SC-HC treatments of the same N rates, but apparent double-crop yield advantages were not statistically significant in any other treatments.

2.4.3 Soil N dynamics

Base soil N concentrations at ROS 2014 were similar across DC SC treatments. In 2015, base soil N following forage bi-culture harvest in DC treatments were 3 and 12 kg N ha^{-1}, compared to 32 and 30 kg N ha^{-1} a LAM 2015 and WAS 2015, respectively (Table 2.6). Fall soil N was significantly affected by N rate at all environments, with crop and interaction effects at LAM 2014 (Table 2.6). Fall N increased significantly with N rate except at ROS 2014, and was greater in DC-HC ($F=34$, $p<0.0001$; $F=17$, $p<0.0001$; $F=4$, $p<0.01$) and DC-SD ($F=22$, $p<0.0001$; $F=16$, $p<0.0001$; $F=7$, $p<0.0001$) than SC-HC ($F=10$, $p<0.0001$; $F=7$, $p<0.0001$; $F=0$, $p>0.9$) at LAM 2014, LAM 2015 and WAS 2015, respectively. Highest concentrations of residual N occurring in DC-SD treatments, although this difference was only significant at LAM 2014, where mean soil N concentrations of DC-SD treatments were 41 and 36% greater than in DC-HC and SC-HC treatments (Table 2.6).

Nitrogen balance was calculated to estimate crop treatment effects on soil N resources. Negative N balance indicates net loss of soil N through crop export, a positive value indicates a net addition of N into soil, and neutral value suggests a balance between N inputs and corn biomass N exports. Nitrogen balance for corn interacted strongly with crop and N rate treatments at all environments (Table 2.7). Nitrogen balance for all crop treatments became more positive as

applied N rate increased. Sole-crop corn consistently assimilated more N than was applied as fertilizer, resulting in negative N balances for SC-HC treatment at all environments. At low N rates, DC-HC treatments N balances were -34 to -71 kg N ha^{-1}. With N224, DC-HC treatments resulted in N surpluses of 52 to 219 kg N ha^{-1} (Table 2.7). Double-crop SD treatments resulted in N surpluses for nearly all N rate treatments.

Crop and N rate treatment effects on residual spring soil N following rye harvest were significant in 2014 environments, but not in 2015. Spring N in DC treatments increased significantly with increasing N rates (Table 2.6). The magnitude of this increase was greatest at LAM 2014, with soil N concentrations increasing by 48 kg N ha^{-1} in DC-SD treatment and 19 kg N ha^{-1} in DC-HC treatments. At ROS 2014, increases of 8 and 6 kg N ha^{-1} for DC-SD and DC-HC treatments, respectively, were also statistically significant. In 2014, spring N in SC-HC treatments ranged from 4 to 11 kg N ha^{-1}, and was similar for all N rates (Table 2.6). In 2015, soil N concentrations were not greater than 6 kg N ha^{-1}, and did not vary across treatments.

2.4.4 Economic analysis

Yield and economic returns were only calculated for DC-HC and SC-HC treatments, due to under-performance of corn in DC-SD treatments. Relative forage quality of forage bi-culture averaged 189 across environments (Table 2.3). Silage yields in DC-HC treatments where calculated at 35% DM, and averaged 18.3 Mg ha^{-1} at LAM and ROS 2014, and 17.5 and 22.6 Mg ha^{-1} at LAM and WAS 2015, respectively. Yields were 38 to 56% of county-wide silage yields reported during the duration of this study. County yields for ROS and WAS were 58 and 59 Mg ha^{-1} in 2014 and 2015, respectively, and yields for LAM were 48 Mg ha^{-1} in 2014 and 56 Mg ha^{-1} in 2015 (NASS, USDA). Nutritive quality parameters of DC-HC silage were similar across all environments. Crude protein, ADF, NDF, and NDFD 48 averaged 7, 32, 53, and 52%, respectively (data not shown). Crude protein increased with N rate significantly ($p < 0.01$) at LAM 2015, but other parameters were unaffected by N rate treatments.

Economic returns for DC-HC and SC-HC were calculated for each system component. Returns from DC forage bi-culture ranged from \$42 ha^{-1} at ROS 2014 to \$1,719 ha^{-1} at WAS 2015 (Table 2.8). Grain and silage returns varied by crop treatment and N rate. In all environments, returns from DC silage corn were significantly less than returns from SC grain corn. Silage production at LAM 2014 resulted in negative returns for all N rates, with losses increasing as N rate increased (Table 2.8). Silage production at ROS 2014 yielded positive returns with all N rates, but in 2015 positive returns were only significantly greater than 0 in N90 treatments at LAM (Table 2.8). Returns from SC grain corn averaged from \$819 ha^{-1} at LAM

2014 to $1,977 at WAS 2015, and increased significantly with N rate at all environments except LAM 2014 (Table 2.8). Maximum returns were achieved in N0, N179, N90, and N179 treatments at LAM 2014, ROS 2014, LAM 2015 and WAS 2015, respectively.

Net total returns were significantly affected by crop treatments at all environments, N rates at all environments excluding LAM 2014, and treatment interactions at ROS 2014 and WAS 2015 (Table 2.8). Positive returns from DC treatments averaged at ROS 2014, LAM 2015, and WAS 2015 were primarily due to the contributions of forage bi-culture, which accounted 94, 63 and 97% of net returns, respectively. While net total returns for DC-HC treatments were significantly lower than those from SC-HC treatments when averaged across N rate, returns from N0-N134 treatments were statistically similar between DC-HC and SC-HC treatments at ROS 2014 and WAS 2015.

2.5 Discussion

Double-crop systems produced statistically similar biomass DM yield to sole-crop systems in most environments, but productivity and profitability of DC systems depended upon crop treatment and environment. The agronomic advantages of double cropping typically accrue from the extension of the growing season to include early spring and late fall (Heggenstaller et al., 2009). Double-cropping frequently results in greater total biomass production than sole-crop systems (Heggenstaller et al., 2009; Jemison et al., 2012). Cool-season cereals and legumes accumulate growing degree units at lower baseline temperatures (Teasdale et al. 2004) than maize (McMaster & Wilhelm, 1997), allowing for increased biomass production in early spring. In 2014, both DC and SC treatments were planted on 5 May, yet DC forage bi-cultures accumulated more GDU during spring than sole-crop hybrid corn (Figure 2.2).

Despite GDU advantage from spring forage, DC-HC treatments did not produce yield advantages in the range ranges of 20 to 33% observed in other studies (Heggenstaller et al., 2008; Jemison et al., 2012), except at ROS 2014 in N0 treatment. This was due to corn yield deficits which were related to GDU reduction and soil moisture deficits. In all environments, SD corn did not approach reported yield potential. Schaefer et al. (2011) reported semidwarf corn DM yield of 10.1 Mg ha^{-1} in Minnesota, which compared to 1.5-2.2 Mg ha^{-1} in the present study. Poor performance of SD corn can be partially explained by agronomic difficulties associated with double-crop system. Despite efforts to ensure weed-free conditions, post-emergence weed control in DC-SD treatments were unsuccessful, and weed pressure negatively impacted SD corn growth at all locations. Reduced growth in hybrid corn is expected with reduced GDU. Darby and Lauer

(2002) reported 9 to 63 % decreases in corn DM biomass when planting date moved from May to late June in Wisconsin, with a maximum decrease of -0.2 Mg ha^{-1} d^{-1} due to delayed planting. Jemison et al. (2012) report an 18% DM yield decline between June and July planting dates in double-crop hybrid corn in New England, and a 30% decline between July-planted double-crop and sole-crop corn. In the present study, DC hybrid corn DM yield decreased by 40 to 60% in response to planting delay of 58 and 104 days compared to SC hybrid corn. GDU reduction was significant enough that DC hybrid corn did not reach physiological maturity. However, the system under investigation in this study utilized spring-planted forage rather than fall-planted cover crops, which allow for corn planting dates as early as 2 June (Heggenstaller et al., 2009; Jemison et al., 2012; Krueger, et al., 2012).

While reduced GDU was the primary limitation on DC corn yield, soil moisture deficits may also have been a factor. Water deficits during vegetative growth have been shown to reduce leaf growth, reducing N-sink strength and limiting N demand (Gonzalez-Dugo and Durand, 2010). Studies have also reported that water deficits can reduce efficiency of N utilization in maize (Di Paolo & Rinaldi, 2008; Széles et al., 2012). Double cropping can limit soil moisture through two mechanisms. First, evapotranspiration of spring crop draws down soil moisture content. In Minnesota, Krueger et al. (2011) found that harvesting rye in immediately prior to corn planting, reduced soil moisture by 16% for a 20 day period. Second, delayed planting date can cause vegetative growth in primary to coincide with periods of low precipitation and exacerbate moisture deficit from spring evapotranspiration. This dynamic may have affected DC-HC treatments at LAM 2014 and ROS 2014, which experienced rainfall deficits of 50 and 70% compared to historical averages in July, respectively (Figure 2.2.1). Moisture deficits may not have affected DM yield of DC-HC treatments at WAS 2015, as above-average rainfall from July through September of 2015 would have maintained adequate soil moisture.

When growth is not limited, N uptake by double-crop corn can approach that of sole-crop corn. Heggenstaller et al. (2008) found that N uptake by sole-crop corn planted in May plateaued approximately 100 days after planting, reaching 152 kg ha^{-1} by October. Despite a 30 day delay in planting, N uptake by June planted double-crop corn overtook sole-crop corn with a yield of 165 kg ha^{-1} by harvest. In the present study, N uptake was limited, and DC treatments showed weaker response to N rate compared to SC-HC treatments.

Predicted optimum N rates for hybrid corn DM yields can range widely across sites and years. Using quadratic plateau models, Nyiraneza et al. (2010) reported optimums ranging from 73 to 235 kg N ha^{-1} in 2007 and 48 to 200 kg N ha^{-1} in 2008. Using both quadratic and quadratic plus plateau models, Qiu et al. (2015) reported a tighter range of optimum N, from 140 to 210 kg

N ha^{-1}. In the present study, ONR for SC-HC DM yield of 293 to 501 kg N ha^{-1} at ROS 2014 and WAS 2015 far exceeded those reported in the literature, and suggest that N availability limited DM yield in SC-HC treatments. However, predicted optimums for DC-HC in the present study agree with the lower range of Qiu et al. (2015), and were consistently within range of applied N rates. Further evidence for N surpluses in DC treatments is provided by residual fall soil N data.

Double-cropping is often associated with a reduction in soil mineral N (Krueger et al., 2011; Heggenstaller et al. 2008). Although DC-HC system was less limited by N inputs than sole-crop system, DC treatments did not compare favorably to SC treatments in terms of N balance. In this study, double cropping did not reduce soil inorganic N compared to sole crop system, except prior to main-crop planting, when double crop plots showed 60 to 97% reduction due to N uptake by forage. While residual fall N was similar between crop treatments, N rate effect was greater in DC treatments (Table 2.6).

Effects of surplus N in DC treatments were observed at rye harvest the following spring. In 2014, late rye planting limited rye establishment, and resulted in low rye yield and N uptake. However, residual spring N was both greater in DC treatments, and had stronger N rate effects than SC-HC treatments (Table 2.6). In 2016, favorable spring conditions led to greater rye yield and N uptake. As a result, N uptake by rye followed patterns expected from calculated N balance (Table 2.7), with high N uptake in DC-SD and DC-HC treatments, and low concentrations of spring soil N (Table 2.6). Ability of double cropping to reduce soil mineral N concentrations and reduce potential N loss through leaching, is thus dependent on N uptake capacity of main crop. If N uptake by primary crop is limited, as in the current study, double-cropping with high rates of N inputs may actually increase risk of N loss.

Although it was hypothesized that spring-planted forage residues might contribute N to DC corn, growth limitation from abiotic factors such as GDU and soil moisture deficits masked any potential benefit from below-ground residue N. Still, a growing body of evidence demonstrates the significance of living plant roots and rhizodeposition for the formation and turnover of labile soil organic matter pools, whose N contents are not estimated using standard tests for mineral N (Cotrufo, et al., 2013; Gardner & Sarrantonio, 2012; Malpassi, et al., 2000; Sheng et al., 2015; Bingham & Cotrufo, 2015; St. Luce, et al., 2016). Root-derived, labile organic N may still play an underappreciated role in N dynamics of double-crop systems, and their role in N uptake in double crop settings warrants further study.

Previous studies have shown mixed results on economic viability of corn silage double-crop systems in the northern latitudes in the continental United States. Thelen & Leep (2002) report lower returns from rye-silage double crop system ($432 ha^{-1}) compared to $538 for sole-

crop corn in Michigan. However, Jemison et al. (2012) found both increased DM yield and forage quality for double-crop corn silage in New England, indicating potential to offset feed costs for vertically-integrated dairy production (Ketterings et al., 2015; Russelle et al., 2007). In the present study, low silage yields treatments meant that economic returns in double cropping derived almost exclusively from spring forage yield. This also means that N rate was not a factor in determining profitability of double-crop treatments. This confirms hypothesis 3, that double-cropping with forage bi-cultures can be optimized with fewer N fertilizer inputs.

Returns from double-crop treatments were derived mostly from spring forage bi-culture. Except at LAM 2014, forage bi-culture yields ranged from 9 to 16 Mg ha^{-1}, within the range of yields reported in other studies (Lithourgidis & Dordas, 2010; Strydhorst et al., 2008). While estimates of economic value where not tied to RFQ in this study, forage bi-culture RFQ of 188 is near upper range of commonly reported forage quality. Premium for forage quality is typically worth $0.82/Mg per point RFQ (Jeranyama & Garcia, 2004; Understander, 2000). This confirms hypothesis 1, that spring annual bi-cultures can be harvested profitably as forage in double-crop systems. Estimated net returns from sole-crop corn in the present study are consistent with five-year state average of $2,057 ha^{-1} (FINBIN). The potential for adequate grain production in double-crop system was demonstrated in central Iowa by Heggenstaller et al. (2008), where triticale-double crop corn produced 8 Mg grain DM ha^{-1}, compared to 10.3 for sole-crop corn. Thelen & Leep (2002) found a reduction of only 1.0 Mg grain DM ha^{-1} in wheat-corn double-crop system in Michigan. In these studies, spring forage crop was planted the previous fall, allowing for earlier primary crop planting date. The late harvest of spring forage in the present study reduced corn GDU, preventing maturation of double-crop corn and reduced silage yield to 50% of county averages. This challenges hypothesis 2; that early maturing corn varieties can produce grain in a double crop. While semidwarf corn growth reached physiological maturity in three of four environments, its growth was severely limited. The 89 RM hybrid variety selected for SC-HC treatments was not appropriate for double-cropping in Minnesota. Regardless of variety selection, double-cropping with spring-planted forages is inadvisable, and future research should consider fall-established forage crops as a necessary component of double-cropping with corn. The present study intended to demonstrate the capacity of double-crop, forage-grain corn systems to provide similar economic value as sole-crop corn using fewer N inputs. Due to reduced growing degree days, the reliably profitable integration of double-crop forage and corn grain production was not demonstrated. However, high yields of spring-planted forage bi-culture did result in similar economic returns between sole-crop and double-crop treatments at Rosemount in 2014. Further, silage production was optimized at low rates of N fertilizer inputs, suggesting that

double-cropping in Minnesota does hold potential as low N input alternative to sole-crop grain production.

2.6 Tables and Figures

Table 2.1. Timeline of planting and harvest dates for sole- (SC) and double- crop (DC) systems at Rosemount (ROS), Lamberton, (LAM) and Waseca, MN (WAS) from 2014-2016.

Year	Location	System	Forage planting date	Forage Harvest Date	Corn planting Date	Corn harvest Date	Rye planting	Rye harvest
2014-2015	LAM	DC	5-May-14	30-Jun-14	3-Jul-14	14-Oct-14	15-Oct-14	2-Jun-15
		SC	--	--	6-May-14	14-Oct-14		
	ROS	DC	5-May-14	30-Jun-14	3-Jul-14	8-Oct-14	15-Oct-14	1-Jun-15
		SC	--	--	6-May-14	8-Oct-14		
2015-2016	LAM	DC	6-Apr-15	24-Jun-14	2-Jul-15	5-Oct-15	12-Oct-15	18-May-16
		SC	--	--	1-May-15	5-Oct-15		
	WAS	DC	2-Apr-15	23-Jun-14	15-Jul-15	13-Oct-15	15-Oct-15	25-May-16
		SC	--	--	17-Apr-15	13-Oct-15		

Table 2.2. Accumulated growing degree units (GDU) at harvest for forage, primary crop, and rye from double-crop (DC) and sole-crop (SC) systems at Rosemount, MN (ROS), Lamberton, MN (LAM) and Waseca, MN (WAS) from 2014-2016.

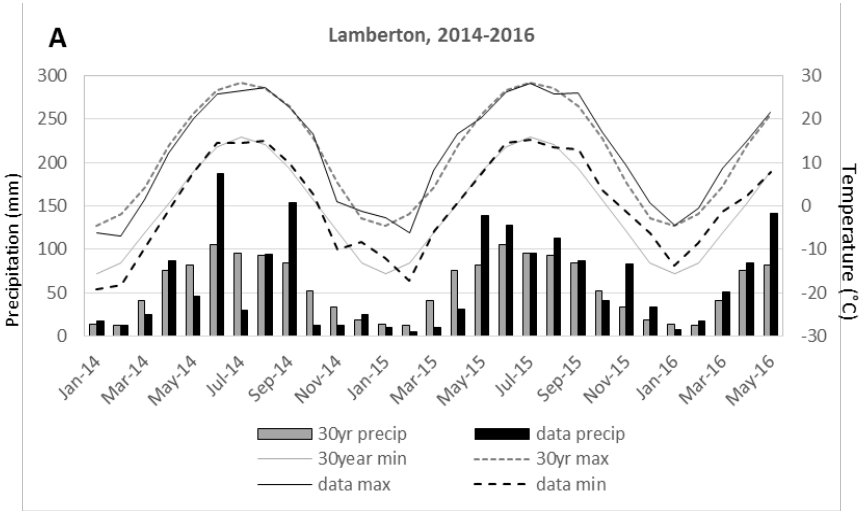
Year	Location	System	Forage	Corn	Main Rotation Total †	Rye fall ‡	Rye spring
			----------GDU----------				
2014-2015	LAM	DC	740	902	1642	156	615
		SC	--	1396	1396		
	ROS	DC	783	992	1775	225	638
		SC	--	1505	1505		
2015-2016	LAM	DC	822	973	1804	240	441
		FSC	--	1513	1513		
	WAS	DC	853	894	1747	112	533
		FSC	--	1560	1560		

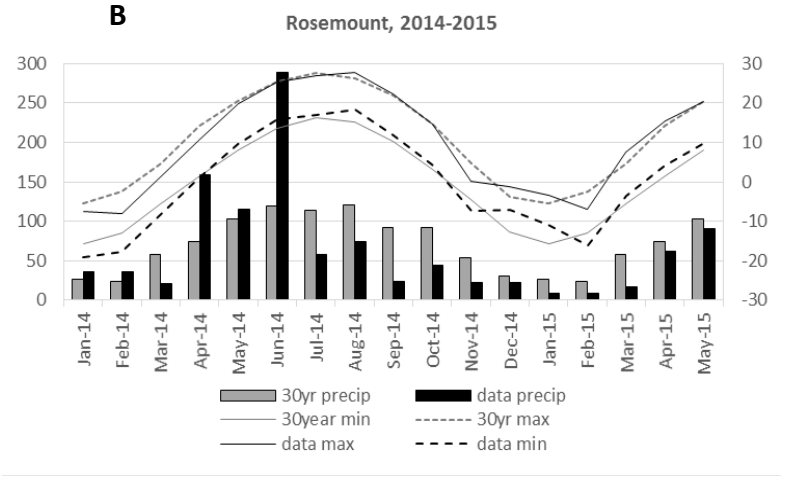
† Main rotation total is sum of forage and main crop GDU.

‡ Rye 'fall' and 'spring' are GDU accumulated by rye following DC and SC fall and spring growing periods, respectively.

Table 2.3. Biomass dry matter (DM) yield, N yield, pea DM as proportion of total DM, crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), neutral detergent fiber digestibility (NDFD 48), relative forage quality (RFQ) and net economic returns of pea-barley forage bi-culture in double-crop semidwarf corn (DC-SD) and hybrid corn (DC-HC) systems from 2014-2015 at Rosemount (ROS) and Lamberton (LAM), and from 2015-2016 in LAM and Waseca (WAS), MN.

		Yield		Proportion of pea DM basis	Forage nutritive parameters					Economic analysis
Year	Location	DM	N yield		CP	ADF	NDF	NDFD 48	RFQ	Net Returns
		--Mg ha^{-1}--	--kg ha^{-1}--	%	--g kg^{-1}--					$ ha^{-1}
2014	LAM	2.9	53	24	114	308	524	613	196	42
	ROS	7.7	143	37	121	317	527	609	189	997
2015	LAM	8.8	113	19	80	315	522	498	190	813
	WAS	13.9	252	16	116	328	514	493	180	1719





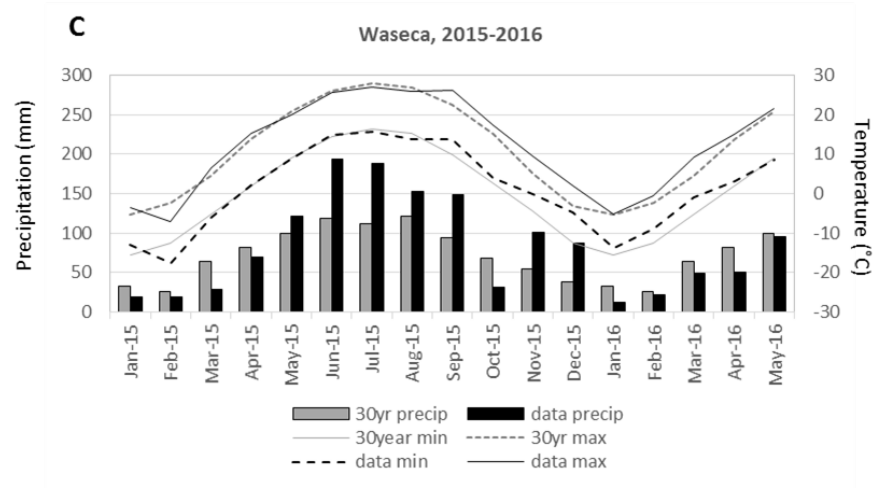


Figure 2.1. Precipitation and temperature at Lamberton, MN from 2014-2016 (A), Rosemount, MN from 2014-2015 (B) and Waseca, MN from 2015-2016 (C).

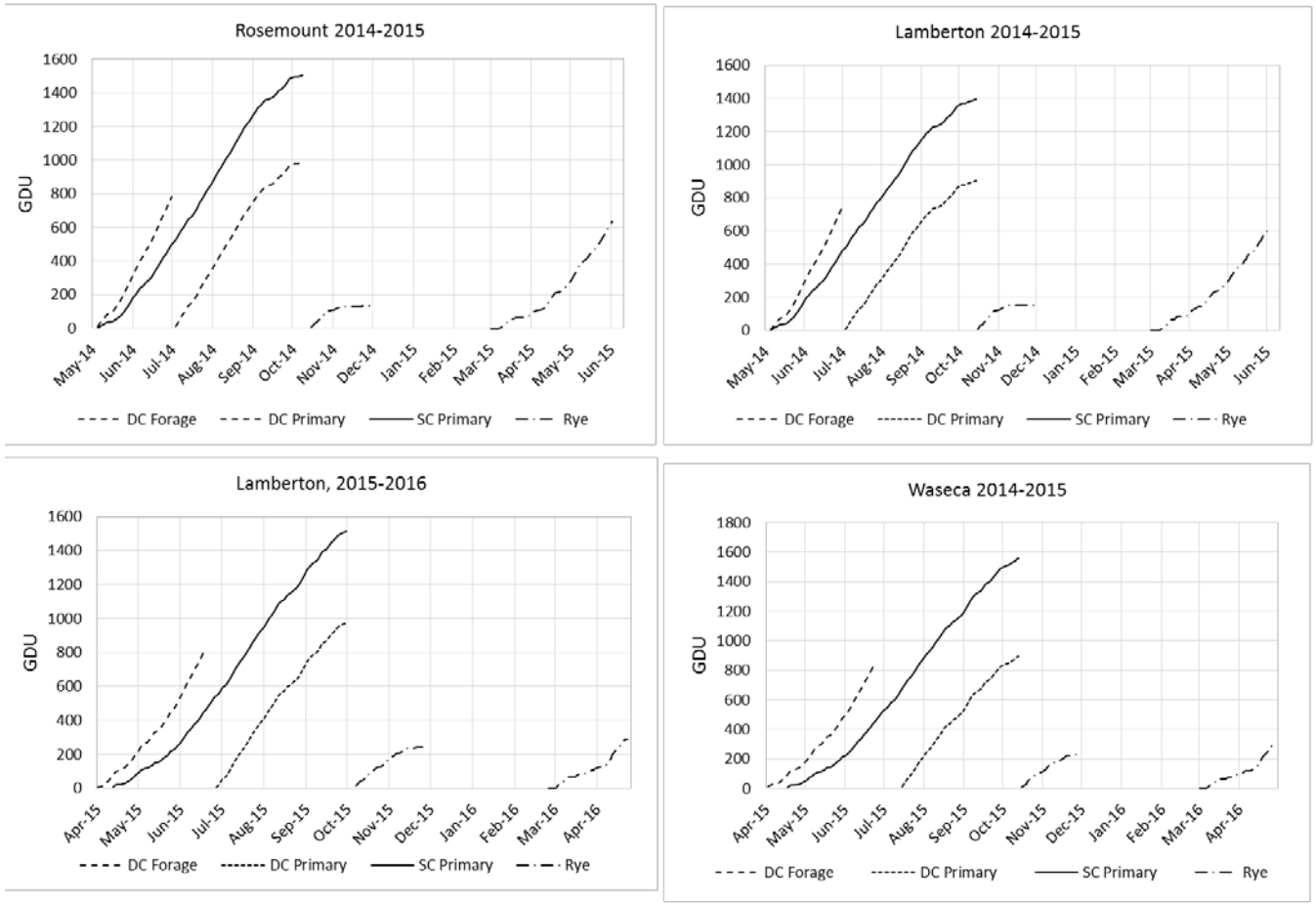


Figure 2.2. Growing degree units (GDU) accumulated by forage, primary crop, rye and rotation totals for double-crop (DC) and (SC) rotations from 2014-2015 in Rosemount and Lamberton, and from 2015-2016 in Lamberton and Waseca, MN.
DC rotation is: DC forage—DC primary—Rye.
SC rotation is: SC primary—Rye. Base GDU for

Table 2.4. Dry matter (DM) biomass yield, corn, rye and system totals for double-crop semidwarf corn (DC-SD) and double-crop (DC-HC) and sole-crop hybrid corn (SC-HC) systems from 2014- at Rosemount (ROS) and Lamberton (LAM), and from 2015-2016 in LAM and Waseca (WAS), MN.

		Corn §				**Rye**				**Total § ¶**			
		---2014---		---2015---		---2014---		---2015---		---2014---		---2015---	
Crop	**N rate**	LAM	ROS	LAM	WAS	LAM	ROS	LAM	WAS	LAM	ROS	LAM	WAS
		Mg ha^{-1}											
DC-HC	N0 †	6.0aA ‡	7.6aA	6.0aAB	5.6bB	1.5aA	2.0aA	2.8aA	1.7aA	10.3aA	17.2aA	16.4bA	20.9abA
	N45	6.3aA	8.7aA	9.1aA	8.0abB	1.5aA	1.8aA	2.6aB	1.9aAB	10.6aA	18.2aA	20.5abA	22.9abA
	N90	5.3aB	10.3aA	12.4aA	6.6abB	1.5aA	2.2aA	2.5aB	1.8aAB	9.7aB	20.2aA	23.7aA	19.2bA
	N134	6.7aA	10.1aB	11.0aA	9.4aB	1.9aA	2.4aA	3.9aB	2.3aAB	11.3aA	20.2aA	23.7aA	24.8aA
	N179	7.1aB	10.9aB	8.3aA	8.7abB	1.8aA	2.7aA	2.7aB	2.1aB	11.6aB	21.3aA	20.0abAB	23.8abA
	N224	6.0aB	9.9aB	11.5aA	7.9abB	1.7aA	2.5aA	3.6aA	2.1aB	10.5aA	20.0aA	24.0aA	24.2abA
	Mean	*6.2b††*	*9.5b*	*9.5b*	*7.6b*	*1.6a*	*2.3a*	*3.0b*	*2.0b*	*10.7b*	*19.5a*	*21.2a*	*22.6a*
DC-SD	N0	1.2bB	2.0aB	1.7aB	1.6aC	1.7aA	2.9aA	3.8bA	2.7aA	5.7aB	12.6aB	14.5aA	16.0aA
	N45	2.3aB	1.8aB	1.6aB	0.9bC	1.9aA	2.6aA	4.6aA	3.1aA	7.0aB	12.0aB	15.0aB	16.4aA
	N90	2.0abC	2.5aB	1.6aB	1.4abC	1.7aA	3.2aA	4.7aA	2.9aA	6.5aC	13.4aB	15.4aB	16.3aA
	N134	1.4abB	2.2aC	1.4aB	1.7aC	1.7aA	3.2aA	5.9aA	3.0aA	5.9aB	13.0aB	16.6aB	16.6aB
	N179	1.2bC	2.2aC	1.8aB	1.4abC	1.5aA	2.6aA	4.8aA	3.6aA	5.5aC	11.9aB	15.7aB	18.6aA
	N224	1.9abC	2.5aC	2.3aB	2.1aC	1.8aA	3.2aA	4.5aA	3.6aA	6.1aB	13.2aB	15.9aB	17.3aB
	Mean	*1.6c*	*2.2c*	*1.7c*	*1.5c*	*1.7a*	*3.0a*	*4.7a*	*3.1a*	*6.1c*	*12.7b*	*15.5b*	*16.8b*
SC-HC	N0	10.2aA	10.4bA	13.3aA	15.4aA	1.4aA	2.2aA	2.3aA	1.8aA	11.6bA	12.8cB	17.2bA	17.2bA
	N45	11.6aA	13.6abA	19.0aA	16.8aA	1.6aA	2.2aA	2.3aB	1.5aB	13.1abA	15.8bcAB	21.3abA	18.3bA
	N90	11.7aA	14.3abA	19.4aA	18.6aA	1.6aA	1.6aA	2.4aB	1.7aB	13.3abA	15.9bcAB	21.7abA	20.3abA
	N134	11.5aA	17.7aA	20.09aA	19.8aA	1.7aA	1.8aA	3.2aB	1.6aB	13.1abA	19.5abA	24.1aA	21.4abAB
	N179	14.5aA	19.7aA	21.0aA	22.6aA	2.4aA	2.3aA	2.5aB	1.7aB	16.8aA	22.0aA	23.5aA	24.3aA
	N224	13.1aA	18.9abA	20.6aA	22.2aA	2.0aA	2.9aA	3.1aA	1.5aB	12.7abA	21.7aA	23.7aA	23.7aAB
	Mean	*12.0a*	*15.4a*	*18.8a*	*19.0a*	*1.8a*	*2.2a*	*2.6b*	*1.6b*	*13.3a*	*17.6a*	*21.8a*	*20.7a*
	Sources of Variation	$F_{(dfn,dfd)}$											
	Crop	$381_{(2,6)}$***	$204_{(2,9)}$***	$79_{(2,9)}$***	$1109_{(2,6)}$***	$0_{(2,6)}$	$3_{(2,9)}$	$49_{(2,6)}$***	$36_{(2,5)}$***	$155_{(2,6)}$***	$26_{(2,9)}$***	$73_{(2,53)}$***	$12_{(2,9)}$**
	N rate	$2_{(5,44)}$	$12_{(5,44)}$***	$2_{(5,44)}$	$10_{(5,36)}$***	$1_{(5,45)}$	$2_{(5,45)}$	$5_{(5,45)}$***	$2_{(5,36)}$	$3_{(5,45)}$*	$13_{(5,44)}$***	$10_{(5,53)}$***	$9_{(5,43)}$***
	Crop x N rate	$3_{(10,44)}$*	$2_{(10,44)}$*	$1_{(10,44)}$	$3_{(10,36)}$**	$1_{(10,45)}$	$2_{(10,45)}$	$1_{(10,45)}$	$2_{(10,36)}$	$4_{(10,44)}$**	$6_{(10,44)}$***	$2_{(10,53)}$	$2_{(10,42)}$*

Table 2.4

*, ** and *** represent significance of *F* tests at α = 0.05, 0.01, and 0.001, respectively.

§ DM yield were e^x transformed to meet ANOVA assumptions.

¶ System total is sum of forage, corn, and rye for DC treatments, and sum of corn and rye for SC treatments.

† Nx: N presents nitrogen, number x presents applied N rate in kg ha^{-1}.

‡ In each environment (year and location), numbers followed by the same lowercase letter are not significantly different within each crop treatment, and numbers followed by the same uppercase letter are not significantly different amongst same N rate treatments between different crop treatments, at *P* = 0.05 by Tukey's HSD Test.

†† Mean DM yield for all N rates within crop treatment. Numbers followed by same lowercase, italicized letter are not significantly different within each crop treatments, at *P* = 0.05 by Tukey's HSD Test.

Table 2.5. N uptake by corn and rye for double-crop semidwarf corn (DC-SD) and double-crop (DC-HC) and sole-crop hybrid corn (SC-HC) systems from 2014-2015 at Rosemount (ROS) and Lamberton (LAM), and from 2015-2016 in LAM and Waseca (WAS), MN.

		Corn §				**Rye**			
		---2014---		---2015---		---2014---		---2015---	
Crop	**N rate**	LAM	ROS	LAM	WAS	LAM	ROS	LAM	WAS
		kg ha^{-1}							
DC-HC	N0†	55aA‡	74bA	23bB	61aA	24aA	27aA	34aA	27aA
	N45	68aB	89abA	65abAB	73aA	31aA	30aA	30aA	28aA
	N90	65aB	118abA	111aA	66aB	34aA	40aAB	30aB	28aA
	N134	77aB	116abB	104aA	89aA	39aA	44aAB	52aB	35aA
	N179	85aB	137aB	84abAB	96aB	39aA	53aA	39aB	41aB
	N224	75aB	117abB	109aAB	92aB	39aA	49aA	52aA	38aAB
	Mean	*70b*	*106b*	*73b*	*75b*	*34a*	*41ab*	*40b*	*33b*
DC-SD	N0	21aB	34aB	25aB	6bB	30aA	45aA	41cA	41bA
	N45	29aC	26aB	22aB	8abB	38aA	49aA	50bcA	48abA
	N90	30aC	43aB	23aB	14aC	42aA	63aA	56abcA	43abA
	N134	26aC	37aC	14aB	13abB	42aA	63aA	80aA	50abA
	N179	21aC	30aC	24aB	9abC	38aA	52aA	67abA	66aA
	N224	22aC	47aC	30aB	15aC	43aA	60aA	63abcA	64abA
	Mean	*24c*	*35c*	*22c*	*10c*	*39a*	*55ab*	*60a*	*52a*
SC-HC	N0	90bA	115cA	135aA	151aA	24aA	35aA	21aA	28aA
	N45	156abA	152bcA	221aA	178aA	27aA	34aA	25aA	27aA
	N90	161abA	144bcA	219aA	204aA	30aA	31aB	30aB	26aA
	N134	169abA	229abA	251aA	234aA	34aA	30aB	31aB	26aA
	N179	222aA	265abA	265aA	265aA	38aA	44aA	28aB	27aB
	N224	194aA	268aA	270aA	272aA	42aA	56aA	42aA	26aB
	Mean	*160a*	*186a*	*221a*	*212a*	*32a*	*39b*	*29c*	*27b*
	Sources of Variation	$F_{(dfn,dfd)}$							
	Crop	$207_{(2,6)}$***	$218_{(2,9)}$***	$48_{(2,6)}$***	$15_{(2,4)}$***	$2_{(2,6)}$	$6_{(2,9)}$*	$59_{(2,54)}$***	$29_{(2,5)}$**
	N rate	$4_{(4,43)}$**	$12_{(5,42)}$***	$4_{(5,43)}$**	$7_{(5,35)}$***	$7_{(5,44)}$***	$6_{(5,45)}$***	$11_{(5,54)}$***	$4_{(5,35)}$**
	Crop x N rate	$9_{(10,43)}$	$3_{(10,42)}$**	$2_{(10,43)}$*	$2_{(10,35)}$	$1_{(10,44)}$	$2_{(10,45)}$	$2_{(10,54)}$	$2_{(10,35)}$

Table 2.5

*, ** and *** represent significance of *F* tests at α = 0.05, 0.01, and 0.001, respectively.

§ N uptake was e^x transformed to meet ANOVA assumptions.

† Nx: N presents nitrogen, number x presents applied N rate in kg ha^{-1}.

‡ In each environment (year and location), numbers followed by the same lowercase letter are not significantly different within each crop treatment, and numbers followed by the same uppercase letter are not significantly different amongst same N rate treatments between different crop treatments, at *P* = 0.05 by Tukey's HSD Test.

†† Mean DM yield for all N rates within crop treatment. Numbers followed by same lowercase, italicized letter are not significantly different within each crop treatments, at *P* = 0.05 by Tukey's HSD Test.

Table 2.6. Soil mineral N concentration prior to corn planting (baseline), following corn harvest (fall) and following rye harvest (spring) in double-crop semidwarf corn (DC-SD), double-crop (DC-HC) and sole-crop hybrid corn (SC-HC) systems from 2014-2015 at Rosemount (ROS) and Lamberton (LAM), and from 2015-2016 in LAM and Waseca (WAS), MN.

	Baseline ¶					**Fall §**				**Spring §**			
	---2014---		---2015---			---2014---		---2015---		---2014---		---2015---	
Crop	LAM	ROS	LAM	WAS	**N rate**	LAM	ROS	LAM	WAS	LAM	ROS	LAM	WAS
	----------kg ha^{-1}----------					----------kg ha^{-1}----------							
DC-HC	--	5	3	12	N0†	4cB‡	18aA	14cdA	10aA	4bA	7bA	4aA	4aA
					N45	11cA	14aA	7dA	7aA	4bA	7bA	5aA	6aA
					N90	32bbA	35aA	19bcdA	8aA	6baA	7bA	4aA	4aA
					N134	33abA	56aA	36abcA	14aA	11abAB	9abA	5aA	5aA
					N179	41abA	28aA	57abA	23aA	10abB	14aA	4aA	6aA
					N224	87aA	59aA	77aA	16aA	23aAB	13abA	5aA	5aA
					Mean	*23b††*	*30a*	*26a*	*12a*	*8b*	*9ab*	*5a*	*5a*
DC-SD	--	6	3	12	N0	15cA	21aA	12cA	9bA	4cA	8bA	4aA	4aA
					N45	25bcA	40aA	16bcA	15bA	7cA	12abA	4aA	3aA
					N90	55abA	37aA	28abcA	14bA	13bcA	10abA	5aA	3aA
					N134	100aA	65aA	53abA	24abA	35abA	10abA	5aA	4aA
					N179	113aA	67aA	83aA	33abA	54aA	14abA	5aA	6aA
					N224	129aA	75aA	101aA	56aA	52aA	16aA	5aA	4aA
					Mean	*56a*	*47a*	*36a*	*21a*	*18a*	*11a*	*5a*	*4a*
SC-HC	37	5	32	30	N0	12cA	30aA	16bA	26aA	4aA	7aA	4aA	4aA
					N45	20bcA	18aA	18bA	25aA	4aA	7aA	5aA	7aA
					N90	23bcA	44aA	19bA	24aA	4aA	7aA	6aA	6aA
					N134	35abA	16aA	35abA	21aA	4aB	8aA	4aA	5aA
					N179	26abcB	30aA	45abA	26aA	7aB	10aA	4aA	5aA
					N224	65aA	49aA	64aA	24aA	9aB	11aA	5aA	4aA
					Mean	*26b*	*29a*	*29a*	*24a*	*5b*	*8b*	*5a*	*5a*
					Sources of Variation	----------$F_{(dfn,dfd)}$----------							
					Crop	$12_{(2,9)}$**	$3_{(2,9)}$	$3_{(2,9)}$	$3_{(2,3)}$	$23_{(2,9)}$***	$8_{(2,50)}$**	$1_{(2,51)}$	$0_{(2,8)}$
					N rate	$59_{(5,45)}$***	$3_{(5,45)}$*	$37_{(5,44)}$***	$6_{(5,35)}$***	$20_{(5,45)}$***	$9_{(5,50)}$***	$1_{(5,51)}$	$2_{(5,40)}$
					Crop x N rate	$4_{(10,45)}$**	$1_{(10,45)}$	$2_{(10,44)}$	$3_{(10,35)}$*	$3_{(10,45)}$**	$1_{(10,50)}$	$0_{(10,51)}$	$1_{(10,40)}$

Table 2.6

*, ** and *** represent significance of *F* tests at α = 0.05, 0.01, and 0.001, respectively.
¶ Baseline soil N measurements were not taken prior to corn planting in DC treatments at Lamberton in 2014
§ Soil N was e^x transformed to meet ANOVA assumptions.
† Nx: N presents nitrogen, number x presents applied N rate in kg ha^{-1}.
‡ In each environment (year and location), numbers followed by the same lowercase letter are not significantly different within each crop treatment, and numbers followed by the same uppercase letter are not significantly different amongst same N rate treatments between different crop treatments, at *P* = 0.05 by Tukey's HSD Test.
†† Mean soil N concentration for all N rates within crop treatment. Numbers followed by same lowercase, italicized letter are not significantly different within each crop treatments, at *P* = 0.05 by Tukey's HSD Test.

Table 2.7. Nitrogen balance for corn in double-crop semidwarf corn (DC-SD) and double-crop (DC-HC) and sole-crop hybrid corn (SC-HC) systems from 2014-2015 at Rosemount (ROS) and Lamberton (LAM), and from 2015-2016 in LAM and Waseca (WAS), MN.

		N Balance			
		---2014---		---2015---	
Crop	**N rate**	LAM	ROS	LAM	WAS
		----------kg N ha^{-1}----------			
DC-HC	N0 †	-57e‡	-71d	-34c	-46e
	N45	-25de	-41cd	-19bc	-14de
	N90	24cd	-24cd	-17bc	36cd
	N134	57bc	20bc	33b	54bc
	N179	93ab	47ab	93a	98ab
	N224	149a	111a	117a	151a
	Mean	*40b*††	*7b*	*29b*	*47b*
DC-SD	N0	-18e	-28d	-24e	6e
	N45	14de	25dcd	25de	48de
	N90	59cd	52c	65cd	88cd
	N134	106bc	102bc	11bc	133bc
	N179	158ab	153ab	152ab	182ab
	N224	199a	190a	191a	219a
	Mean	*86a*	*82a*	*88a*	*113a*
SC-HC	N0	-54bc	-118a	-146c	-122c
	N45	-75c	-107a	-104bc	-104bc
	N90	-36bc	-62a	-100bc	-85bc
	N134	-5b	-96a	-90bc	-71b
	N179	-9b	-47a	-56ab	-57ab
	N224	61a	-49a	-17a	-20a
	Mean	*-19c*	*-80c*	*-85c*	*-77c*
	Sources of Variation	----------$F_{(dfn,dfd)}$----------			
	Crop	$76_{(2,6)}$***	$69_{(2,9)}$***	$373_{(2,51)}$***	$425_{(2,7)}$***
	N rate	$103_{(5,44)}$***	$45_{(5,44)}$***	$89_{(5,51)}$***	$117_{(5,35)}$***
	Crop x N rate	$4_{(44,3)}$***	$5_{(10,44)}$***	$6_{(10,51)}$***	$7_{(10,35)}$***

Table 2.7

*, ** and *** represent significance of *F* tests at α = 0.05, 0.01, and 0.001, respectively.

† Nx: N presents nitrogen, number x presents applied N rate in kg ha^{-1}.

‡ In each environment (year and location), numbers followed by the same lowercase letter are not significantly different within each crop treatment at *P* = 0.05 by Tukey's HSD Test.

†† Mean value for all N rates within crop treatment. Numbers followed by same lowercase, italicized letter are not significantly different within each crop treatments, at *P* = 0.05 by Tukey's HSD Test.

Table 2.8. Net economic returns of corn and total net returns in double-crop semidwarf corn (DC-SD) and double-crop (DC-HC) and sole-crop hybrid corn (SC-HC) systems from 2014-2015 at Rosemount (ROS) and Lamberton (LAM), and from 2015-2016 in LAM and Waseca (WAS), MN.

		Corn Returns				**Total Returns ¶**			
		---2014---		---2015---		---2014---		---2015---	
Crop	**N rate**	LAM	ROS	LAM	WAS	LAM	ROS	LAM	WAS
		$ ha^{-1}							
DC-HC	N0 †	69aB‡	217aB	-85bB	31aB	111aA	1,030aA	951bA	1,750aA
	N45	-4aB	231aB	264abB	152aB	37aB	1,044aA	1,260abB	1,833aA
	N90	-174aB	347aB	574aB	-41aB	-133aB	1,160aA	1,570aA	1,689aA
	N134	-100aB	267aB	347abB	177aB	-58aB	1,080aA	1,344abB	1,897aA
	N179	-104aB	288aB	46bB	52aB	-63aB	1,101aB	1,043abB	1,758aB
	N224	-277aB	120aB	291abB	100aB	-235aB	933aB	1,288abB	1,709aB
	Mean	*-99b††*	*245b*	*240b*	*79b*	*-99b*	*1,058b*	*1,243b*	*1,773b*
SC-HC	N0	798aA	1,156cdA	1,394bA	1,600cA	798aA	931dA	1,394aA	1,600cA
	N45	794aA	1,103dA	1,866abA	1,704bcA	794aA	1,103cdA	1,866aA	1,704bcA
	N90	899aA	1,283bcA	1,922aA	1,966abA	899aA	1,283bcdA	1,922aA	1,966abcA
	N134	730aA	1,721abA	2,087aA	2,076abA	730aA	1,721abA	2,087aA	2,076abA
	N179	1065aA	1,927aA	1,951aA	2,274aA	1,065aA	1,927aA	1,951aA	2,274aA
	N224	629aA	1,690abA	1,937aA	2,243aA	8,84aA	1,690abcA	1,937aA	2,243aA
	Mean	*819a*	*1,480a*	*1,860a*	*1,977a*	*862a*	*1,443a*	*1,860a*	*1,977a*
	Sources of Variation			$F_{(dfn,dfd)}$					
	Crop	$77_{(1,3)}$**	$287_{(1,3)}$***	$556.6_{(1,7)}$***	$1206_{(1,32)}$***	$130_{(1,33)}$***	$14_{(1,6)}$**	$60_{(1,6)}$***	$15_{(1,33)}$***
	N rate	$2_{(5,29)}$	$6_{(5,29)}$***	$7_{(5,29)}$***	$5_{(5,32)}$*	$1_{(5,33)}$	$6_{(5,30)}$***	$7_{(5,30)}$***	$5_{(5,33)}$**
	Crop x N rate	$1_{(5,29)}$	$6_{(5,29)}$***	$2_{(5,29)}$	$5_{(5,32)}$*	$1_{(5,29)}$	$6_{(5,30)}$***	$2_{(5,30)}$	$6_{(5,33)}$***

*, ** and *** represent significance of *F* tests at α = 0.05, 0.01, and 0.001, respectively.

¶ Total returns are sum of net returns from forage, and corn yield components in DC treatments, and from corn in SC treatments.

† Nx: N presents nitrogen, number x presents applied N rate in kg ha^{-1}.

‡ In each environment (year and location), numbers followed by the same lowercase letter are not significantly different within each crop treatment, and numbers followed by the same uppercase letter are not significantly different amongst same N rate treatments between different crop treatments, at *P* = 0.05 by Tukey's HSD Test.

†† Mean returns for all N rates within crop treatment. Numbers followed by same lowercase, italicized letter are not significantly different within each crop treatments, at *P* = 0.05 by Tukey's HSD Test.

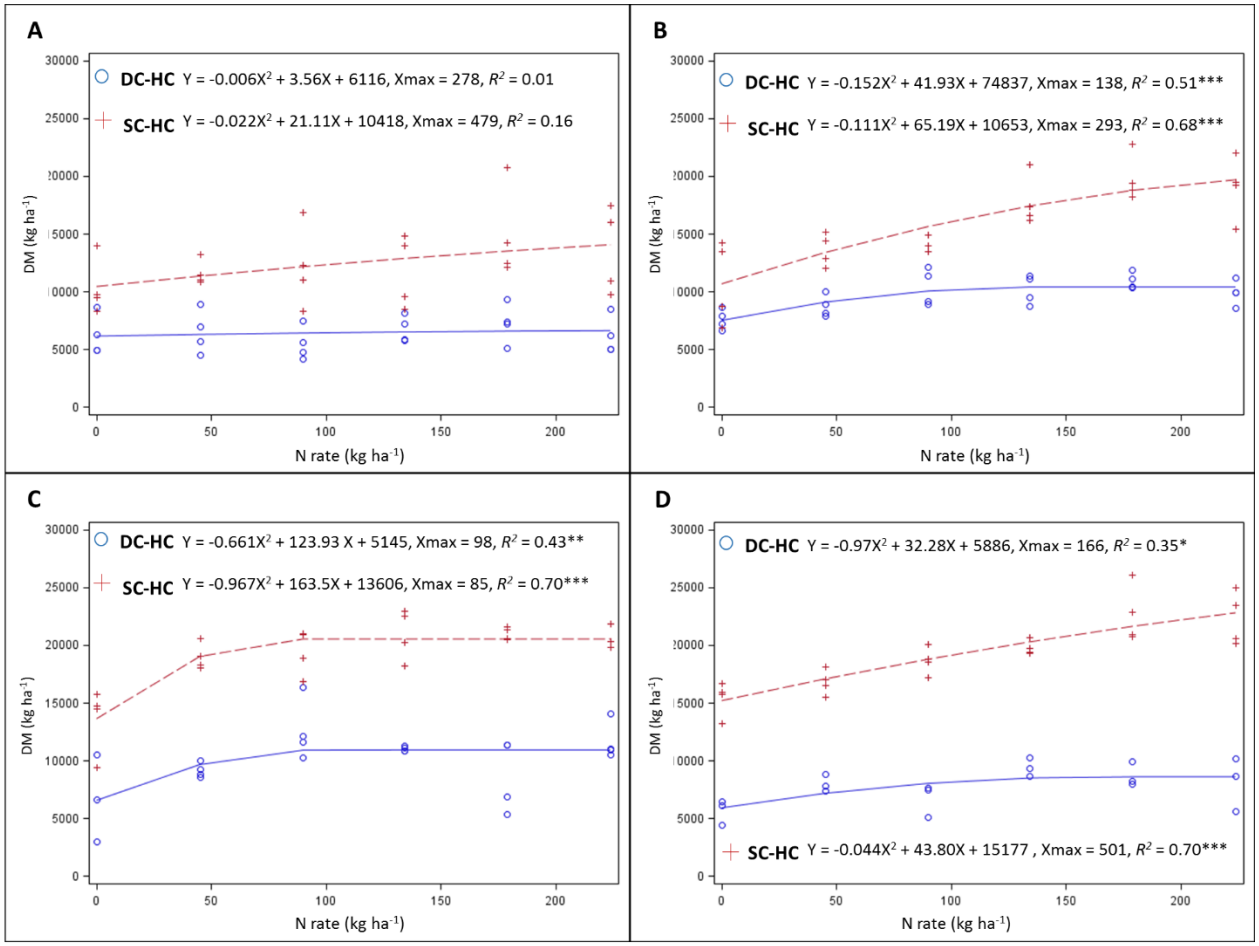


Figure 2.3. Dry matter (DM) yield of corn as a function of N fertilizer rate in double-crop hybrid corn (DC-HC) and sole-crop hybrid corn (SC-HC) rotations from 2014 in Lamberton (A) and Rosemount (B), and from 2015 in Lamberton (C) and Waseca (D), in Minnesota. Predicted optimum N rate (Xmax) was calculated using quadratic plus plateau model.
*, **, and *** represent significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively

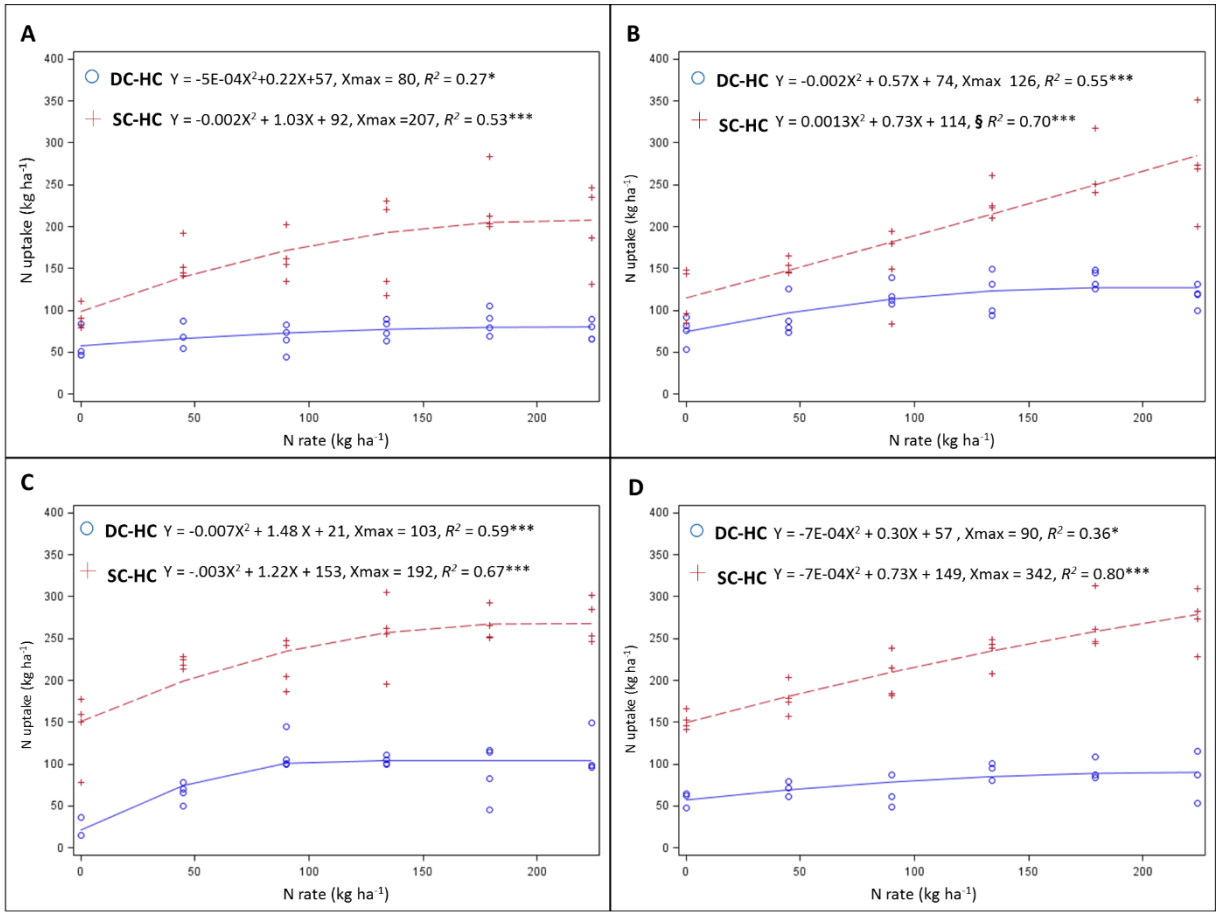


Figure 2.4 uptake of corn as a function of N fertilizer rate in double-crop hybrid corn (DC-HC) and sole-crop hybrid corn (SC-HC) rotations from 2014 in Lamberton (A) and Rosemount (B), and from 2015 in Lamberton (C) and Waseca (D) in Minnesota. Predicted optimum N rate (Xmax) was calculated using quadratic plus plateau model.

*, **, and *** represent significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively

§ . B: SC-HC was fit using a quadratic model.

3 Factors affecting the distribution of ^{15}N label in legume roots in *in situ* isotopic labeling studies: A research proposal

3.1 Introduction

The nitrogen (N) contributions of below-ground legume biomass (BGN) are significant, poorly understood, and frequently underestimated fraction of soil N budget. BGN is the sum of root biomass N and N rhizodeposition (Wichern, et al. 2008). In the past two decades, many attempts have been made to quantify BGN by labeling plants with ^{15}N. In isotopic labeling studies, accurate estimates of rhizodeposition rely on the satisfaction of two conditions: the relatively equal distribution of ^{15}N throughout measured plant parts, and that $\%^{15}N$ in rhizodeposition is equivalent to that of (Wichern et al., 2008). Unfortunately, most labeling methods do not result in homogenous partition of ^{15}N (Rasmussen et al., 2013; Rasmussen, 2011; Wichern et al., 2008). There are two primary approaches: Natural assimilation pathways (such as atmospheric or root labeling), or induced labeling (shoot labeling). Although labeling through natural assimilation pathways offer homogenous labeling of plant parts and high uptake compared to induced pathways (Wichern et al., 2011), they are difficult to adopt in field conditions. Consequently, shoot-labeling techniques have been the most widely used in recent years.

Labels can be applied either continuously, or in pulses. In pulse labeling, photosynthates assimilated at the time of labeling are more enriched than older or new ones. Therefore, estimates of rhizodeposition from pulse labeling events are not representative of the entire growth period. Applications of multiple pulses can improve diminish this effect, but continuous labeling ensures that concentrations of isotopic label in plant parts and root exudates are more homogenous and representative of plant growth.

The two primary methods for shoot-labeling are the wick method for stem labeling, and leaf-tip immersion. Both are appropriate for *in situ* labeling studies under field conditions, and high ^{15}N uptake has been reported with both methods. Leaf immersion is less invasive than stem labeling, and is somewhat simpler to apply. ^{15}N can easily be applied with multiple pulses. However, tracer losses frequently occur, and overall plant enrichment is highly variable with leaf position, urea concentration, and growth condition (Wichern et al., 2008). Leaf labeling results in preferential enrichment of leaves compared to other shoot-labeling methods (Khan et al., 2002; Yasmin et al., 2006).

Stem labeling with the wick method offers a few distinct advantages over leaf labeling: improved ^{15}N uptake (Hertenberger & Wanek, 2004; C a Russell & Fillery, 1996), precise measurement of ^{15}N-urea uptake (Arcand et al., 2013), and capacity for continuous labeling (Arcand et al., 2013b; Mahieu et al., 2009). It has been claimed that continuous labeling can result in more homogenous distribution of ^{15}N throughout plant parts (Arcand et al., 2013a; Mayer el al., 2003). Experimental evidence for this claim is

limited (Mahieu et al. 2009; Russell and Fillery, 1996). In single or double- pulse studies, leaf labeling has resulted in more even enrichment than stem-labeling (Yasmin, 2006). However, continuous labeling does provide more accurate estimates of rhizodeposition (Mahieu et al. 2009). More-ever, these two methods have only been directly compared in two studies (Hertenberger and Wanek, 2004; Yasmin et al. 2006)

Both methods result in heterogeneous distribution of ^{15}N label, with above-ground plant biomass accumulating a greater proportion of ^{15}N than roots (Khan et al., 2002a; Khan et al. 2002b; Mahieu et al., 2009; Rasmussen et al., 2013; Wichern et al., 2011). More-ever, ^{15}N partitioning within root systems may also be highly uneven (Rasmussen et al. 2013). In particular, nodulated roots are typically less ^{15}N enriched than non-nodulated roots, as atmospheric N_2 dilutes the tracer (Arcand et al., 2013b; Khan et al., 2002; Mahieu et al., 2007). This may explain some of the ^{15}N enrichment heterogeneity seen across rooting depth in some studies (McNeill, et al. 1997; Russell & Fillery, 1996). However, Arcand et al. (2013b) noted that ^{15}N enrichment of nodules increased with root age under continuous labeling, which may be accounted for by the decrease in N_2 fixation as plants mature.

While heterogeneous ^{15}N enrichment of plant components can be accounted for using a tracer mass-balance approach, it is still necessary to understand factors that influence ^{15}N distribution throughout plant components. Unfortunately, detailed studies on factors affecting below-ground ^{15}N partitioning are lacking.

This pilot will compare the effect of shoot labeling techniques and frequency on ^{15}N uptake and enrichment of hairy vetch. Continuous wick-labeling will be compared to pulse labeling using both the wick method and leaf immersion. Additionally, the enrichment of below-ground plant parts will be recorded in greater detail than has been reported in the literature by isolating fine roots, coarse roots, nodules. Since BGN of hairy vetch has not yet been studied in the literature, this study will pave the way for new methods of studying its contributions to soil N pools. By addressing the need for thorough accounting of ^{15}N tracer distribution throughout plant parts, and by directly comparing two commonly used labeling methods, it will increase general knowledge about isotopic tracer methods and their relative utility for measuring below-ground legume N.

3.2 Methods

3.2.1 Plant growth and experimental design

Two legume species, hairy vetch (*Vicia villosa* L. Roth) and kura clover *(Trifolium ambiguum*), will receive three labeling treatments (Stem-wick continuous, stem-wick fortnightly, or leaf-immersion fortnightly) at five repetitions per treatment. Five unlabeled natural abundance control plants for each species will be also be grown, for a total of 35 experimental units. Plants will be grown in a 50/50 soil/sand mixture, grown in 20cm x 50cm (0.2 m^3) pots at a density of one plant per plot. Pots will be lined with plastic to facilitate root harvesting and prevent isotopic loss. Legumes will be inoculated with

appropriate, commercially available rhizobia species. Pots will be watered regularly to maintain approximately 60% field capacity. Labeling for all treatments will commence at roughly the 4 leaf unfolding stage (~3 weeks after planting), and continue until plants reach physiological maturity.

3.2.2 ^{15}N labeling

Plants will be supplied with ^{15}N from a 0.5 % (w/v) solution of ^{15}N-enriched (99atom %) urea, using either the wick method (Russell and Fillery 1996; Mayer et al. 2003) or the leaf-immersion method (McNeill et al. 1997). For the cotton wick method, a cotton wick is passed through a hole in the stem, drilled with a 0.5 mm drill at crown of plant. The cotton wick run through silicone tubing, is inserted into a 1mL vial containing the solution. Placticine seals connections between the wick and plant stem to prevent transpiration losses. Plants will be labeled either continuously (every 1-4 days) or biweekly using a sterile syringe. For the leaf immersion technique, tips (1-2mm) of 1 leaf per plant are cut and immersed in 0.5 ml urea solution in a 2 ml vial. Leaf-labeling will occur on a bi-weekly basis, as continuous labeling is not possible using leaf-feeding techniques. In both treatments, after completion of solution uptake, vials will be filled with ~0.3 ml of deionised water to rinse vials of remaining urea to maximize total ^{15}N uptake by plants. Fallen leaves will be collected in a mesh fixed around the pot to prevent isotopic contamination of soil. Applied urea solution volume and concentration will be calculated based on N demand curve of vetch, estimated to maintain 2.5% ^{15}N excess in plant (Mayer et al. 2003; Arcand et al. 2013a,b).

3.3.3 Soil and Plant Samples

At physiological maturity (or flowering), above-ground plant biomass will be harvested and separated into leaves, stems, pods, and seeds, weighed and dried at 60 C to constant weight. Intact roots and visible roots fragments will be separated from soil with a 2-mm sieve and tweezers. Roots will be gently rinsed, and resulting soil slurry will be collected and oven dried at 75 C to recover soil. Root biomass will be separated into coarse roots (>1 mm diameter), fine roots, (<1 mm diameter) and nodules, weighed, and dried at 60 C to constant weight. All plant and soil samples will be weighed and ground. Total N content and ^{15}N:^{14}N ratio will be analyzed using mass spectrometry.

3.3.4 Calculations

^{15}N enrichment (%^{15}N atom excess) is determined by the difference of atom% ^{15}N of recovered sample and atom% ^{15}N of natural abundance control. Soil N derived from rhizodeposition (%NdfR) is calculated using the equation (Janzen & Bruinsma, 1989):

$$\%NdfR = (\text{atom } \%^{15}N_{soil} - \text{atom}\%^{15}N \text{ background}_A) / (\text{atom } \%^{15}N_{roots} - \text{atom}\%^{15}N \text{ background}_B) \times 100$$

where $background_A$ and $background_B$ are natural abundance atom% N of soil and roots, respectively, of unlabeled plants (Schmidtke, 2005).

3.3 Protocol: Stem-wick method for labeling legumes using ^{15}N-enriched urea

Purpose

To label plant biomass with ^{15}N isotope *in situ* using ^{15}N-enriched urea. The stem wick method allows for continuous isotope enrichment and precise measurement of ^{15}N uptake. This protocol is an expansion of a protocol developed by Arcand (2015) at the University of Saskatchewan.

3.3.1 Materials

^{15}N-enriched urea solution:

- Urea: >99.0 atom % ^{15}N enriched
- Mortar and pestle
- Beaker for stock solution
- Beaker for dilute solution
- Parafilm.

Labeling:

- Wick: 100% cotton sewing thread (synthetic thread will not wick solution)
- Tube: 0.76 mm inner diameter (ID) silicon tubing
- Vials: 2 ml gas spectrometry (GS) vials, clear
- Caps with septa (12 x 32 mm screw thread)
- Needle: <0.5 mm diameter (beading needle works well; gauge must be large enough for thread)
- Plasticine (modeling clay) or plumber's putty
- Drill bit: <0.5 mm diameter
- Beaker(s) for solution
- Beaker for DI water
- Beaker for waste solution
- Parafilm

- Syringe and needle: 1 ml (single use, sterile)
- Scissors
- Forceps

3.3.2 Prepare ^{15}N enriched stock solution urea solution

- If study utilizes multiple concentrations of ^{15}-N enriched urea solution, it will help to mix a concentrated solution. Before every labeling event, smaller quantities of desired solutions can be made from stock.
- Autoclave deionized water for stock ^{15}N-enriched urea solution
- Under flow hood, weigh quantity of ^{15}N-enriched urea pellets required to mix stock solution of desired concentration.
- Grind urea using mortar and pestle. Mix urea in deionized water.
- Seal beaker with cap and store in fridge.
- To mix dilute concentrations from stock, dilute desired volume of stock solution in autoclaved, deionized water under flow hood into beaker with cap.

3.3.3 Prepare Materials

- Cut crescent wedge out of each septa. This allows silicon tube to be threaded silicon tubing into vial.

  (Figure 2.3.1)
- Cut thread at equal lengths of 4-6 inches and thread needles. Pre-threading needles in advance saves time.
- Cut tubing into equal lengths of 2-4 inches. You will require two lengths of tube per stem.
- Autoclave threaded needles, vials, caps, septa, and tubing.

3.3.4 Installation

- If labeling branching plant (Hairy Vetch):

    - Wipe soil away from base of stems to expose crown.
    - Drill 0.5 mm hole through crown.
- If labeling single-stem plant (Winter Pea):
    - Drill 0.5 mm hole through stem 2-3 cm from soil surface.
- Pass thread and needle through one length of tubing.
- Pass thread and needle through hole in stem; do not pull thread completely through hole (Figure 2.3.2)
- Pass thread and needle through second length of tubing. Pull tubing flush with stem; there should be one length of tubing on one side of stem, and another length of tubing on the other. (Figure 2.3.3)
- Mold clay into two medium-size thickness discs. Holding tubes flush with stem, place one disc behind stem and press gently. Stem and tubing should form a cross-shape embedded in disc. Mold second clay disc onto stem, and seal as thoroughly as possible. Gaps between stem, tubing, and clay can dry out thread and prevent wicking. (Figures 3.4 and 3.5)
- Thread both ends of tubing through cap of vial. Use forceps ensure that threads reach bottom of vial. Use forceps to place septa into cap, with tubing passing through gap in septa. (Figure 2.3.6)
- Before labeling, unscrew cap from solution beaker and replace with parafilm
- To label, take up 0.1 – 0.5 ml solution through parafilm using sterile needle and syringe.
- Use sterile needle and syringe to inject desired volume of urea solution into vial through septa.
- To re-use syringe, flush out with fresh sterilized, DI water. Store flushed water a separate beaker, and dispose elsewhere to avoid isotopic contamination. Otherwise, dispose of syringe and use another.

3.3.5 Monitoring

- Check on plants frequently and monitor solution uptake. Timing will depend on labeling frequency.

- Solution uptake rate may be measured by extracting remaining solution into a sterile syringe, recording uptake, and re-injecting solution back into vial.
- If solution uptake ceases, wick may have to be re-installed, as plant will create scar tissue surrounding wound, and preventing solution uptake.
- Re-apply solution at desired frequency. Record volume and concentration of solution at every application.
- When watering, be careful to not flood pot. Vial should never be submerged, and submersion of wick-stem connection should be avoided. When wick is connected to crown, as with hairy vetch, temporary submersion is inevitable. Make sure clay seal is secure, and water carefully.

3.3.6 Figures

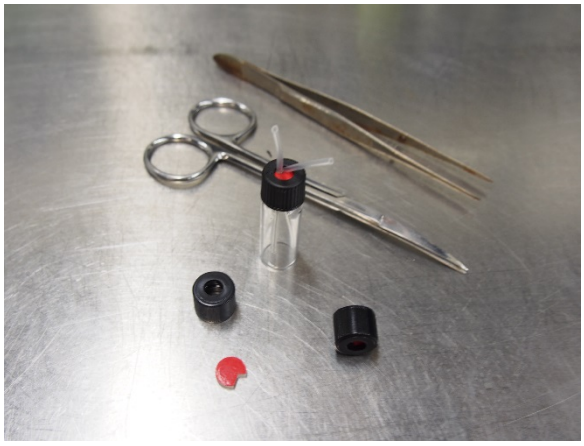


Figure 3.1 Vial, caps, and modified septa.

Figure 3.2 Thread is through one length of tubing (left) and stem of plant

Figure 3.3 Thread is through stem and two length of tubing.

Figure 4 Plasticine is molded around one side of tubing and stem.

Figure 3.5 Plasticine is fully molded around tubing and stem.

Figure 3.6 Tubing is threaded through hole in cap with septa, and thread reaches bottom of vial.

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