NITROGEN CYCLING IN AGROECOSYSTEMS:
THE EFFECTS OF SOIL FERTILITY AND PLANT SPECIES INTERACTIONS ON LEGUME NITROGEN FIXATION

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by
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Meagan Erin Schipanski, Ph. D.
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Legume-based cropping systems have the potential to improve nitrogen (N) retention and use efficiency in comparison with fertilizer-based systems, yet we lack an ecological understanding of biological nitrogen fixation (BNF) in these agroecosystems. My research objectives were to investigate: 1) the effects of legume-based management on soil organic matter (SOM) pools; 2) the effects of a SOM-based fertility gradient on BNF; and 3) how plant phenology and species interactions affect BNF across the fertility gradient.

Research plots were established on grain farm fields in New York in 2004 and 2006. Fields represented a fertility gradient due to soil type and management differences, ranging from exclusive use of Haber-Bosch N to almost exclusive use of legumes. I estimated BNF of soybean (*Glycine max*), field pea (*Pisum sativum*), and perennial red clover (*Trifolium pratense*) using the ^{15}N natural abundance method. Soil N pools were quantified ranging in microbial accessibility from extremely labile to primarily recalcitrant pools.

Legume-based systems had greater quantity and quality of labile SOM pools, compared to fertilizer-based systems. I found weak evidence of soil N availability inhibiting BNF despite a more than 2-fold range in SOM pools across study sites. This suggests that N mineralization from SOM pools represented a much smaller N flux than N fertilizer levels used in past BNF studies.

Complementary, facilitative and competitive interactions influenced BNF in species mixtures. Relay cropping of frost-seeded clover into winter grains increased clover % N from fixation due to the short period of competitive species interactions while retaining high biomass production due to the period of monoculture growth following grain harvest. The longer growth period of perennials may have increased their ability to respond to environmental conditions through feedback mechanisms. Perennial mixtures outyielded their corresponding monocultures and I found evidence for facilitative N transfer from legumes to grasses in perennial, but not annual mixtures. Monoculture red clover biomass yield was more stable across field sites than the other plant treatments. These results suggest that the integration of perennials and species mixtures into rotations could increase BNF inputs while also improving overall agroecosystem N cycling efficiency and yield stability.

BIOGRAPHICAL SKETCH

Meagan was born and raised in Manhattan, Kansas. She received her Bachelors of Arts in Biology from Oberlin College in Oberlin, Ohio, and she spent a semester studying agricultural development in Kenya with the School for International Training. During her undergraduate years, she spent summers working as a field ecology research assistant on the Konza Prairie in Kansas, and in the Sylvania Wilderness Area of the Upper Peninsula of Michigan. Seeking a more direct application of her work, she then spent five years farming, including an internship on Whistling Duck Farm in southern Oregon, and four years as field manager at Angelic Organics farm in Illinois. After burning out on farming, she spent a couple of years working in Seattle before returning to graduate school at Cornell University to study agroecology.

For Nick and Nadia

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CHAPTER 1

UNDERSTANDING THE VARIABILITY IN SOYBEAN NITROGEN FIXATION ACROSS AGROECOSYSTEMS

Abstract

Legume-based cropping systems have the potential to internally regulate N cycling due to the suppressive effect of soil N availability on biological nitrogen fixation. We used a gradient of endogenous soil N levels resulting from different management legacies and soil textures to investigate the effects of soil organic matter dynamics and N availability on soybean (*Glycine max*) N_2 fixation. Soybean N_2 fixation was estimated on 13 grain farm fields in central New York State by the ^{15}N natural abundance method using a non-nodulating soybean reference. A range of soil N fractions were measured to span the continuum from labile to more recalcitrant N pools. Soybean reliance on N_2 fixation ranged from 36% to 82% and total N_2 fixed in aboveground biomass ranged from 40 to 224 kg N ha^{-1}. Soil N pools were consistently inversely correlated with % N from fixation and the correlation was statistically significant for inorganic N and occluded particulate organic matter N. However, we also found that soil N uptake by N_2-fixing soybeans relative to the non-nodulating isoline increased as soil N decreased, suggesting that N_2 fixation increased soil N scavenging in low fertility fields. We found weak evidence for internal regulation of N_2 fixation, because the inhibitory effects of soil N availability were secondary to the environmental and site characteristics, such as soil texture and corresponding soil characteristics that vary with texture, which affected soybean biomass, total N_2 fixation, and net N balance.

Introduction

Humans have more than doubled the global rate of reactive nitrogen (N) input into terrestrial systems, resulting in major direct and indirect effects on ecosystems (Vitousek et al. 2002a; Galloway et al. 2003). Haber-Bosch N (HBN) fertilizer is the primary contributor to this increase and its application contributes to large environmental consequences, including surface water eutrophication and the formation of the annual hypoxic zone in the Gulf of Mexico (McIsaac et al. 2001; Galloway and Cowling 2002). The advent of HBN fertilizers increased the quantity of N additions and also reduced the occurrence of carbon (C) additions in conjunction with N. As a result of this uncoupling of C and N cycles, HBN fertilizers are needed at levels that saturate agroecosystems to maintain net primary productivity (NPP) (Woodmansee 1984; Drinkwater and Snapp 2007). Soluble HBN fertilizers are readily lost from agricultural fields as they are typically added when plant uptake is low and residual HBN fertilizer often is lost due to the absence of active plant growth following annual crop harvest.

Studies of agroecosystems with increased temporal plant diversity, which reduce bare fallow periods and rely primarily on biological N_2 fixation (BNF) for N inputs, show that yields can be maintained while N losses are reduced, through the recoupling of C and N cycles (Drinkwater et al. 1998; Gregorich et al. 2001; Ross et al. 2008). This improved N use efficiency is due in part to significant reductions in the magnitude of the surplus N additions and the addition of N in more stable, organic forms that are mineralized through microbially-mediated processes (Drinkwater et al. 1998). Despite the potential for legume-based systems to improve N use efficiency, scant research has focused on understanding the ecology of BNF in temperate, legume-based cash-grain agroecosystems.

Legume-based cropping systems may have the advantage of a built-in internal feedback that regulates N cycling. As soil organic matter (SOM) levels increase in legume-based systems over time, the mineralization of N from larger SOM pools may suppress BNF (Waterer and Vessey 1993). In natural systems, soil N availability is one of the primary factors thought to constrain the distribution of legumes (Vitousek et al. 2002b). Rhizobial strains differ in their N_2 fixation efficiency and effectiveness, but the plant partner in the symbiosis appears to be the primary regulator of the suppressive effect of soil N availability on BNF (Kiers et al. 2003).

It is not clear whether the inhibitory effect of soil N derived from SOM mineralization is a functionally important mechanism that regulates BNF in agroecosystems. The effect of N availability on legume N_2 fixation has rarely been researched within the context of cropping systems in which soil N availability is dependent on the mineralization of SOM. Studies have typically used large N fertilizer additions, ranging from 50-200 kg N ha^{-1} added at one time point, effectively flooding the root zone with nitrate (Salvagiotti et al. 2008). These studies clearly demonstrate that when soil conditions are held constant, large pools of inorganic N inhibit BNF and variations in N availability act as the major regulator of N_2 fixation rates. However, these studies do not provide an analysis of the relative importance of inorganic N availability in legume-based agroecosystems dependent on the complex plant-soil-microbe interactions that influence N mineralization from heterogeneous SOM pools (Clarholm 1985; Hodge 2003). Nitrate can have a localized as well as a systemic effect on BNF (Streeter 1985; Blumenthal et al. 1997) and extremely low concentrations of inorganic N have even been found to increase N_2 fixation in some greenhouse studies (Gan et al. 2004). Nitrogen mineralization from heterogeneous SOM pools would be expected to create temporally variable microsite hotspots of inorganic N throughout the rooting zone. This is in contrast to the potential influence of the broad application of HBN fertilizers at rates that typically saturate the root system for a short, concentrated time period.

Most soybeans grown in the U.S. do not receive direct HBN fertilizer additions during their growth and are, therefore, dependent on residual HBN fertilizer in the soil and on N mineralization from SOM pools for soil N uptake. In this study, we identified a gradient of endogenous soil N levels across several grain farms in New York State. Our objective was to investigate whether soybean BNF was regulated by N availability from a typical range of SOM levels found across agroecosystems.

Materials and Methods

Experimental sites

We established research plots in 2004 in 13 fields on 4 commercial grain farms in central New York state (42° 39’-42° 44’ N and 77° 04’-76° 43’ W). All fields were within 30 km of each other. The climate is characterized as humid temperate with a mean annual precipitation of 880 mm and mean annual maximum and minimum air temperature of 14°C and 3°C, respectively. We selected fields to reflect a gradient of management histories (Table 1.1). All soils are moderately well to well drained, mixed, active, mesic Hapludalfs, except for fields H4 and L6, which fall into soil classes that are somewhat poorly drained (USDA-NRCS SSURGO database). Weather data were averaged from the Penn Yan Airport, Penn Yan, NY (within 10 km SW of fields L1-L3, and all HBN fields) and the Aurora Research Farm, Aurora, NY (within 15 km NE of fields L4-L8) provided by the National Climate Data Center.

We collected a 5-year cropping history from participating farmers including all N inputs and crop yields for each field. Literature values and data from several years of cover crop and grain N sampling in the region were combined with farmer records of field inputs and exports to calculate estimated N balances for each field. These

Table 1.1. Management history, crop rotation history, and USDA soil classification for 13 study fields.

Field[a]	Years under organic mgmt	5-year rotation history[b]	Soil classification
H1	0	corn-corn-corn-corn-kidney bean	Fine sandy-loam, Glossic Hapludalf
H2	0	corn-corn-kidney bean-rye-corn	Fine sandy-loam, Oxyaquic Hapludalf
H3	0	corn-corn-corn-corn-corn	Fine sandy-loam, Glossic Hapludalf
H4	0	corn-snap bean-wheat/clover-corn-soybean	Silt loam, Aeric Endoaquept
H5	0	kidney bean-wheat/clover-corn-corn-soybean	Fine sandy-loam, Oxyaquic Hapludalf
L1	3	soybean-oat-soybean-corn-soybean	Silt loam, Glossic Hapludalf
L2	8	soybean-spelt/clover-soybean-corn-soybean	Silt loam, Glossic Hapludalf
L3	9	kidney bean-soybean-wheat/clover-clover-soybean	Silt loam, Glossic Hapludalf
L4	5	spelt/clover-corn-wheat/clover-corn-soybean	Silt loam, Oxyaquic Hapludalf
L5	7	corn-soybean-spelt/clover-corn-soybean	Silt loam, Oxyaquic Hapludalf
L6	13	wheat/clover-soybean-spelt/clover-corn-soybean	Silt loam, Aeric Endoaqualf
L7	20	corn-soybean-rye-spelt/clover-soybean	Gravelly silt loam, Glossaquic Hapludalf
L8	20	wheat/clover-corn-wheat/clover-corn-soybean	Gravelly silt loam, Glossaquic Hapludalf

[a] Fields are labeled by management type (H= Haber-Bosch N fertilizer-based; L= legume N-based management practices)
[b] Crop rotation history is listed from least to most recent from left to right, ending with the 2004 season. Corn (*Zea mays*); kidney bean and snap bean (*Phaseolus vulgaris*); rye (*Secale cereale*); wheat (*Triticum aestivum*); spelt (*Triticum spelta*); clover (*Trifolium pratense*)

balances were used to classify each field based on management history and the percentage of total N inputs derived from either HBN fertilizers or legume BNF inputs. The fields used in this study are all cash-grain operations and do not have livestock integrated into their farming systems. The fields that are managed with legume-based rotations apply an average of only 17 kg N ha^{-1} $year^{-1}$ from compost or manure inputs, and are therefore reliant on BNF for the majority of total N inputs. Nitrogen management categories were defined as follows: 1) HBN fields received mainly fertilizer N (>50% of total N inputs, remainder from legume-based BNF) while 2) LEG fields did not receive any HBN, but relied on legume-based BNF for most of their N additions (>50% of N inputs from BNF with remainder from compost or manure). All LEG fields were located on certified organic farms and had been under organic management regimes for 3 to 20 years (Table 1.1).

Plot establishment and management

Research plots were established within grain fields that were managed using conventional tillage practices. The surrounding field area was planted by the farmer to soybean, with the exception of fields H1, H2, and H3 which were in kidney beans or corn in 2004 (Table 1.1). We planted three varieties of soybean in a randomized complete block design with four replicate blocks in each field. We used a late Group I nodulating soybean (M129 Nod) bred from an Altona x Chippewa cross because a non-nodulating isoline (M129 Non-Nod) was available to serve as a reference plant for the BNF estimation. M129 seed was provided by James H. Orf, University of Minnesota. To simulate the farmer practice in these farming systems we also planted Vinton 81, a high protein, food-grade late Group I soybean commonly grown by organic farmers in the region, because of its high market value. We seeded the plots in June 2004 at the rate of 26 seeds m^{-1}, 0.76 m row spacing, in 3 m by 2 m plots (4 rows $plot^{-1}$). Seeds of the two N_2-fixing varieties (Vinton 81 and M129 Nod) were inoculated with commercial soybean inoculum. In fields where the surrounding field was not planted to soybeans, an additional buffer row of soybeans was planted on the outside edges of the research plot area and an additional 2 m was seeded on either end of research plot rows to reduce any shading effect particularly in corn fields (fields H2 and H3). Farmers did not apply fertilizers or amendments to the research plot area during the 2004 growing season. We supplemented farmers' mechanical cultivation with hand-weeding to control weeds in research plots.

Plant sampling and analysis

We collected soybean aboveground biomass samples at early pod formation (R4) and at full pod prior to leaf senescence (R6) from 0.5 m of the center two rows in each plot for each sampling, leaving a 0.5 m buffer between sampling areas (Fehr et al. 1971). Biomass was dried at 60° C and weighed. Dried plant biomass samples were first coarsely ground using both a hammer mill and grinder and then finely pulverized using a roller grinder. Samples were analyzed for ^{15}N natural abundance and total N content using a continuous flow Isotope Ratio Mass Spectrometer (Stable Isotope Facility, UC Davis).

We collected nodules at the first (R4) sampling time when nodules should be fully established, but prior to their senescence (Zapata et al. 1987). Roots from 20 cm of the center two rows were excavated to 25 cm depth. Soil was gently shaken off and roots were rinsed briefly in water in the field and then stored at 4° C. Within 2 weeks of sampling, we separated nodules from roots and counted them. Roots and nodules were dried and weighed. Root sampling allowed for confirmation of nodulation status of non-nodulating soybeans. Only 1.5% of all non-nodulating plants sampled contained nodules (sample size = 454 plants). One plot of non-nodulating soybeans in field L4 had nodulated plants throughout and was obviously misplanted. This plot was dropped from all analyses.

Nitrogen fixation

We used the ^{15}N natural abundance method (Shearer and Kohl 1986) to estimate BNF at the two sampling times. The percentage of N derived from the atmosphere in the N_2-fixing soybean (Vinton 81 or M129 Nod) biomass was calculated using the M129 Non-Nod as the reference plant:

$$\% \text{ N from fixation} = 100*((\delta^{15}\text{N Non-Nod} - \delta^{15}\text{N}_2\text{-fixing})/(\delta^{15}\text{N Non-Nod} - B))$$

where B is the δ^{15}N value of soybean grown with atmospheric N_2 as the only source of N after accounting for seed N. Total aboveground N_2 fixed was calculated from the aboveground dry matter of N_2-fixing soybean, its biomass N concentration, and % N from fixation.

We grew soybeans in N-free, autoclaved, calcined clay media (Turface®, AIMCOR, Deerfield, IL) in a greenhouse to quantify the B value for Vinton 81 and M129 Nod varieties. Seeds were surface sterilized in 70% (v/v) ethanol for 3 minutes and 3% (v/v) bleach solution for 2 minutes, followed by a 3 minute rinse in deionized water. Sterilized seeds received the same inoculant used in field plots and plants were fertilized with an N-free Hoagland's nutrient solution (GreenCare Fertilizers, Chicago, IL). Plants were sampled at the same maturity stages as field study, dried, ground, and analyzed for δ^{15}N. Seeds for each variety were also analyzed for δ^{15}N to allow calculation of seed N contribution.

Description of soil N fractions measured

Despite decades of research, a single soil test that reliably predicts plant available N has not been developed. In order to capture the range of soil N pools that can contribute to plant available N, we quantified a range of soil N fractions spanning the continuum from labile to more recalcitrant N pools (Table 1.2). Different soil N fractions interact with environmental factors to determine soil N availability. Inorganic N is an ephemeral soil N pool with high temporal and spatial variability, because it depends on inputs from microbially-mediated mineralization and losses from denitrification, volatilization, leaching, and plant and microbial immobilization. Microbial biomass (MB) is sensitive to temperature and moisture availability and represents an important sink, as well as a source, for inorganic N depending on C availability. Organic matter can be further partitioned into physical fractions to separate more labile pools from older, recalcitrant pools. Light fraction particulate organic matter (fPOM), also referred to as free POM because it is not associated with soil aggregates, is derived from recent litter inputs and can represent a sink or a source for inorganic N, depending on the source material C:N ratio and biochemistry (Boone 1994; Wander 2004). Occluded particulate organic matter (oPOM), is physically protected within soil aggregates and is more likely than fPOM to be a net source of inorganic N because it typically is more decomposed, resulting in a lower C:N ratio (Wander 2004; Marriott and Wander 2006).

Soil sampling and analysis

We collected soils from all of these fields in September 2004 in conjunction with the second biomass sampling. Soil measurements vary greatly in their sensitivity to seasonality. Key factors that contribute to seasonal shifts in more labile N pools include recent residue inputs, plant cover, soil moisture, and temperature (Bonde and Roswall 1987; Ross 1987). We chose to sample soils after a season of soybean to provide a consistent recent plant influence across all fields, thereby reducing the short-term influence of differences in the previous year's crop. In addition, fall sampling occurred after the cessation of active plant N uptake. Labile soil N pools measured in the fall were, therefore, influenced by recent soybean C and N inputs as well as longer-term management histories and soil textural differences.

Twenty soil cores (2 cm diameter by 20 cm depth) were taken and composited from each plot within each field. For N mineralization potential (N min), a subsample

Table 1.2. Soil N fractions measured, their relative turnover times, and descriptions of which soil N pools they represent.

Soil N fraction	**Estimated turnover time**	**Pool description**	**Source**
Extractable NO_3^- and NH_4^+ (Inorganic N)	Hours to days	Inorganic N immediately available for plant or microbial uptake	Corre et al. 2002
Chloroform extractable N (MBN)	Days to years	Microbial biomass N	McGill et al. 1986; Schmidt et al. 2007
Dissolved organic N (DON)	Days to decades	Heterogeneous pool of labile and more recalcitrant organic molecules	Jones et al. 2004; von Lutzowa et al. 2007
N mineralization potential (N min)	*	Integrated measure of microbial activity and labile N availability	Drinkwater et al. 1996
Light fraction POM N (fPOM N)	Weeks to years	Recent plant and animal residue inputs	Boone 1994; Wander 2004
Occluded POM N (oPOM N)	Years to decades	Partially decomposed residues, particularly root residues, physically protected within soil aggregates	Gale et al. 2000; Wander 2004
Total soil N (Soil N)	Up to centuries to millenia	Majority of pool in recalcitrant mineral-associated compounds	Stevenson 1994

[*] *N min represents an integrated rate of labile N availability rather than a distinct pool with a turnover time.*

of soil was sieved in the field for inorganic N and 7-day anaerobic N mineralization incubation using a 2 *M* KCl extraction (Drinkwater et al. 1996). Total NH_4^+ and NO_3^- were analyzed using a continuous flow analyzer (AlpKem, OI Analytical, College Station, TX). Remaining sieved and unsieved soils were stored at 4° C. Dissolved organic C and N and microbial biomass extractions for each plot were completed within 1 to 3 wk of field sampling on field moist samples using the chloroform fumigation-extraction method (24 h fumigation and 30 min extraction with 0.5 *M* H_2SO_4) as in Horwath and Paul (1994). Samples were lyophilized and analyzed for total N using a continuous flow Isotope Ratio Mass Spectrometer (Stable Isotope Facility, UC Davis). Inorganic N values were subtracted from total dissolved N to calculate dissolved organic N.

Gravimetric water content was recorded for samples and remaining soil was air-dried. We separated fPOM and oPOM using size and density separation as in Marriott and Wander (2006). Briefly, fPOM was separated by floating on sodium polytungstate (1.7 g cm^{-3}). The remaining soil sample was shaken with 10% sodium hexametaphosphate to disperse soil aggregates and then rinsed through a 53 μm filter. The fraction larger than 53 μm, which included sand and particulate organic matter, was retained. Total C and N of POM fractions and total soil were measured using a LECO 2000 CN Analyzer (Leco Corporation, St. Joseph, MO). To correct for carbonate content of each soil, a subset of samples from each field was treated with 6 *M* HCl and then analyzed for total C (Midwood and Boutton 1998). Carbonate C, the difference between untreated and HCl treated soil C, was subtracted from total soil C to calculate total soil organic C content for each field.

Replicate composite samples of air-dried soil sieved to 2 mm for each field were analyzed for Morgan-extractable P, K, Ca, Mg, Cu, Zn, Fe, Al, and Mn (Cornell

Nutrient Analysis Laboratory, Ithaca, NY) and particle size (Agricultural Analytical Services Laboratory, Penn State University, University Park, PA).

Statistical analysis

We computed statistics using JMP v.7 and SAS v.9.1 software (SAS Institute Inc., Cary, NC). Variables were assessed for normal distribution. Nitrogen mineralization potential, oPOM N, fPOM N, and inorganic N were log-transformed to fit a normal distribution. Data were analyzed using mixed models including field and replicate as nested random factors. Least squares means from mixed models were used for comparisons between varieties, sampling time points, and fields. Multiple comparisons were calculated using Tukey's HSD and pairwise comparisons were calculated using Student's *t*-tests.

Field means were used to calculate simple correlations using Pearson correlations and for multivariate forward stepwise regressions. If variety was not a significant predictor ($p>0.05$), the two varieties were assumed to have a similar response to soil variables and values were averaged across both field and variety.

Due to the multicollinearity of the soil variables measured, we used principal components analysis (PCA) to generate independent variables that represent a composite of several soil variables. Principal components analysis was conducted using the SAS PRINCOMP procedure and soils data from plots of both N_2-fixing varieties were used for the PCA. Variables for PCA were selected in two ways. From the paired soil N and C pools, only the soil N pools were included. All soil nutrient variables and soil texture (sand, clay) were included. Variables with low loadings (<0.30) across all components were dropped from the analysis (O'Rourke et al. 2005). Principal components with eigenvalues greater than 1 were retained.

Stepwise regressions were conducted using the SAS REG procedure. Multicollinearity of the selected variables was assessed using condition indices and variance proportions. If the condition index was greater than 30 and variance proportion for two variables was greater than 0.50 in regression results, the variables were considered to be collinear (Freund and Littell 2000). Collinear variables were dropped sequentially from the model to determine which variable explained more of the total variability in the response variable. Non-collinear variables selected using stepwise regressions on field average values were then used in mixed models including all N_2-fixing soybean plots. Variety was included as a fixed effect in regression models to determine whether M129 Nod and Vinton 81 BNF variables differed in response to soil variables. The percentage of variation explained by each fixed effect in multivariate mixed models was calculated as the proportion of the variance component explained by adding each individual fixed effect relative to the variance component explained by the model containing only random effects (Snijders and Bosker 1999).

Results

Weather

The climate during the 2004 growing season was slightly cooler and wetter than historical averages. The temperatures in 2004 followed the 30-year average with the exception of cooler temperatures at the beginning of the growing season (Figure 1.1a). Annual precipitation was 910 mm, which was slightly above normal due to higher precipitation during the beginning and end of the growing season (Figure 1.1b).

Soil analysis

The fields we used for our experimental plots were selected to represent a fertility gradient due to their wide range in SOM content. Total soil N varied more than two-fold across the fields from 0.9 to 2.3 mg N g soil^{-1} (Figure 1.2). Occluded POM N had the widest range of variation across fields of all soil variables measured,

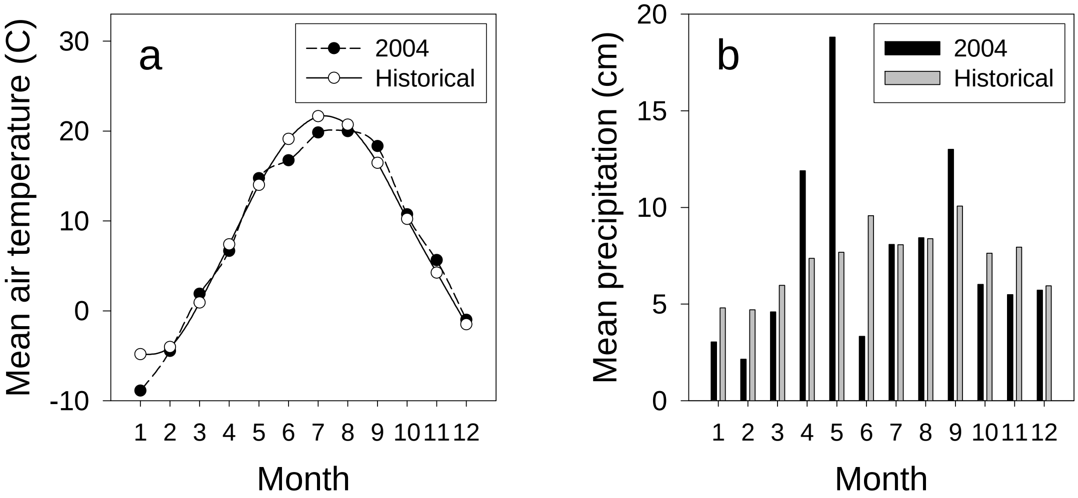
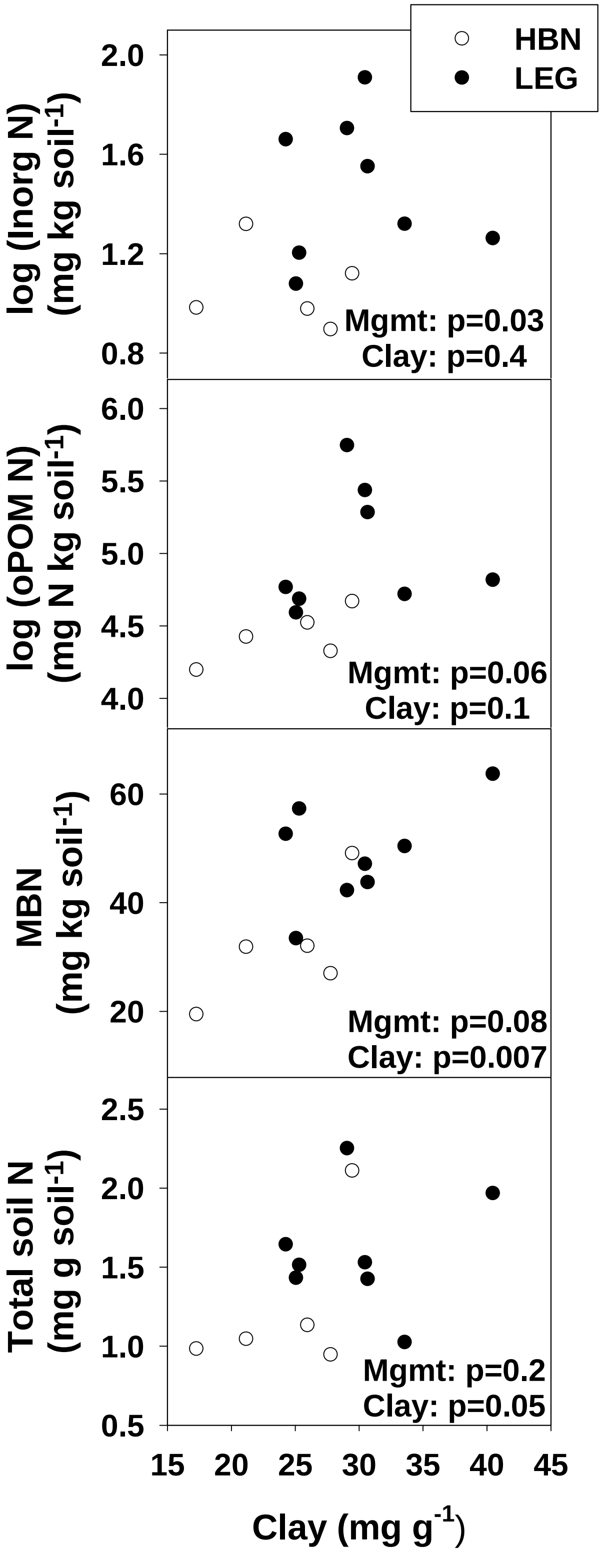


Figure 1.1. Average monthly a) temperature and b) precipitation sums for 2004 and 30-year historical averages for Penn Yan and Aurora, New York.

Figure 1.2. Correlations between soil clay concentration and N pools across 13 farm fields. *P*-values represent significance test results from covariate analysis of management type effects on soil N pools (total soil N, microbial biomass N (MBN), occluded particulate organic matter N (oPOM N), and inorganic N (extractable NO_3^- and NH_4^+)), including soil clay concentration as a covariate. HBN= Haber-Bosch N fertilizer-based management; LEG= legume-based N management (LEG).



HBN
LEG
log (Inorg N)
(mg kg soil^{-1})
2.0
1.6
1.2
0.8
Mgmt: p=0.03
Clay: p=0.4
log (oPOM N)
(mg N kg soil^{-1})
6.0
5.5
5.0
4.5
4.0
Mgmt: p=0.06
Clay: p=0.1
MBN
(mg kg soil^{-1})
60
40
20
Mgmt: p=0.08
Clay: p=0.007
Total soil N
(mg g soil^{-1})
2.5
2.0
1.5
1.0
0.5
Mgmt: p=0.2
Clay: p=0.05
15
20
25
30
35
40
45
Clay (mg g^{-1})

ranging from 68 to 328 mg N kg soil^{-1}. Inorganic N ranged from 2.3 to 9.6 mg N kg soil^{-1} across the fields and differed by soybean variety. Inorganic N in M129 Non-Nod plots was lower than in the two N_2-fixing varieties ($p<0.0001$). Only the inorganic N data from the M129 Non-Nod plots, which ranged from 2.5 to 6.8 mg N kg soil^{-1}, was used to represent inorganic N pools in all analyses (Figure 1.2).

Soil N pool sizes were influenced by a combination of soil texture and management history. Soil clay concentration was greater in LEG fields compared to HBN fields ($p=0.09$) and, as a result, soil texture is confounded with management regime, making it necessary to use covariate analyses to assess management effects on soil properties. The LEG fields tended to have larger labile N pools (Figure 1.2). Covariance analysis using clay as the covariate indicated that management history explained a significant proportion of the variance for inorganic N ($p=0.03$) and oPOM N ($p=0.06$). The variance in MBN and total N was correlated with soil texture (Figure 1.2). When controlling for soil clay content, LEG fields had lower pH and higher N min than HBN fields (Table 1.3).

Soybean varietal characteristics

The two N_2-fixing soybean varieties were similar for most plant variables measured. The M129 Nod and Vinton 81 varieties were similar in total aboveground biomass and total N, supporting the use of the M129 Non-Nod as a reference plant for both varieties (Table 1.4). The % N from fixation did not differ between varieties at early pod formation (R4), but was greater in the Vinton 81 variety by full pod formation (R6). Varietal differences for N_2 fixation variables were primarily due to the different *B* values and the higher N concentration of the food-grade, high protein Vinton 81 soybeans (Table 1.4). Both the specific number and mass of nodules measured at R4 were greater for M129 Nod compared to Vinton 81 even though % N from fixation and total N_2 fixed did not differ by variety at R4 (Table 1.4). As

Table 1.3. Subset of soil properties measured for each field and results for analysis of management effects on soil variables. Fields are labeled by management type (H= Haber-Bosch N fertilizer-based; L= legume N-based management practices).

Field	**Sand**	**Clay**	**pH**	**P**	**K**	**Ca**	**fPOMN**[a]	**DON**[b]	**N min**[e]
	— g kg^{-1} —			——— mg kg^{-1} ———			— mg kg $soil^{-1}$ —		mg N kg $soil^{-1}$ $week^{-1}$
H1	470	260	7.37	9.4	90	1880	27	10.0	3.8
H2	400	280	7.57	3.9	84	3610	19	8.7	6.1
H3	480	210	7.04	8.7	120	1530	24	10.0	10.1
H4	380	290	7.49	9.0	64	3160	27	7.4	5.4
H5	510	170	6.12	5.3	51	816	34	13.5	11.2
L1	480	250	6.79	3.7	78	1810	32	8.1	9.1
L2	400	240	7.35	11.3	60	2710	25	14.3	15.2
L3	480	250	6.49	1.7	47	1660	25	7.8	6.8
L4	310	340	6.81	6.0	45	1590	35	9.7	8.1
L5	400	300	6.56	3.6	47	1780	33	12.6	9.3
L6	270	400	7.23	16.9	53	2960	34	10.7	5.2
L7	390	310	6.60	7.4	42	1900	35	8.9	7.5
L8	330	290	6.37	20.1	86	2440	38	11.3	8.1
Significance test of management effect, controlling for textural differences[d]									
p value			0.04	ns	ns	ns	ns	ns	0.05

[a] free particulate organic matter N; [b] dissolved organic N; [c] N mineralization potential;
[d] Soil clay content included as a covariate in models to test soil variable difference between fields under Haber-Bosch N-based and legume N-based management practices

Table 1.4. Average shoot biomass, shoot N, shoot N concentration, biomass $\delta^{15}N$, *B* values (shoot $\delta^{15}N$ of legumes grown in N-free media), specific nodule number and weight, N_2 from fixation and N assimilated from soil for all soybean varieties at early pod-fill (R4) and full pod-fill (R6).

	R4			**R6**		
	Vinton 81	**M129 Nod**	**Non-Nod**	**Vinton 81**	**M129 Nod**	**Non-Nod**
Aboveground biomass (Mg ha^{-1})	1.3 b[a]	1.4 a	0.9 c	5.3 a	5.3 a	3.2 b
Aboveground total N (kg N ha^{-1})	41 a	41 a	22 b	185 a	172 a	62 b
Aboveground N concentration (mg g^{-1})	3.19 a	2.90 b	2.43 c	3.51 a	3.23 b	1.91 c
Aboveground $\delta^{15}N$ (‰)	3.22 a	3.07 a	6.06 b	0.43 a	0.54 a	4.32 b
B value (‰)	-2.28 a	-2.70 b		-1.98 a	-2.26 b	
# of nodules (# g $root^{-1}$)	73 a	86 b				
nodule weight (mg g $root^{-1}$)	180 a	200 b				
% N from fixation	34 a	34 a		62 a	57 b	
N_2 from fixation (kg N ha^{-1})	16 a	14 a		116 a	98 b	
N from soil (kg N ha^{-1})	26 ab	27 a	22 b	69 a	74 a	62 a

[a] Different letters within rows indicate significant differences by variety at each sampling ($p<0.05$).

expected, aboveground biomass and total N were significantly lower for the non-nodulating M129 isoline than for the N_2-fixing varieties at both sampling times.

^{15}N natural abundance

The ^{15}N natural abundance method relies on the natural ^{15}N enrichment of soil N pools relative to atmospheric N_2 to distinguish between N sources. The δ^{15}N signatures of the N_2-fixing and non-nodulating soybeans showed clear separation and the magnitude of the difference ranged from 2.2 ‰ to 6.7 ‰ across the fields at R6 (Figure 1.3). The δ^{15}N natural abundance signature of M129 Non-Nod soybeans varied across the 13 fields over time. Average δ^{15}N of the non-nodulating soybean biomass decreased between R4 and R6 ($p<0.0001$). The δ^{15}N of the two nodulating soybean varieties did not significantly differ at either sampling point. The significant positive correlations between % N from fixation at R4 and specific nodule mass for both soybean varieties support our ^{15}N natural abundance results ($r=0.67$, $p=0.01$ for M129, and $r=0.78$, $p=0.002$ for Vinton 81).

Biomass and BNF variability across fields

Biomass and BNF varied considerably across the 13 fields. At R4, field average % N from fixation varied 4-fold, ranging from 12 % to 63 % (data not shown). By R6, the variation in the relative reliance on N_2 fixation ranged from 36 % to 82 % (Figure 1.4). Total N_2 fixed had wider variation, from 40 to 224 kg N ha^{-1}, and was strongly correlated with aboveground biomass (Table 1.5).

Total N uptake by the non-nodulating soybean provides one indicator of soil N availability across fields. Soil N uptake by the non-nodulating soybean varied more than 4-fold across the fields from 28 to 116 kg N ha^{-1} (data not shown). At R6, average soil N uptake between the N_2-fixing and non-fixing varieties did not differ (Table 1.4), however, the N_2-fixing soybean varieties accumulated more soil N compared to the non-nodulating soybean in several fields. The difference in soil N uptake by

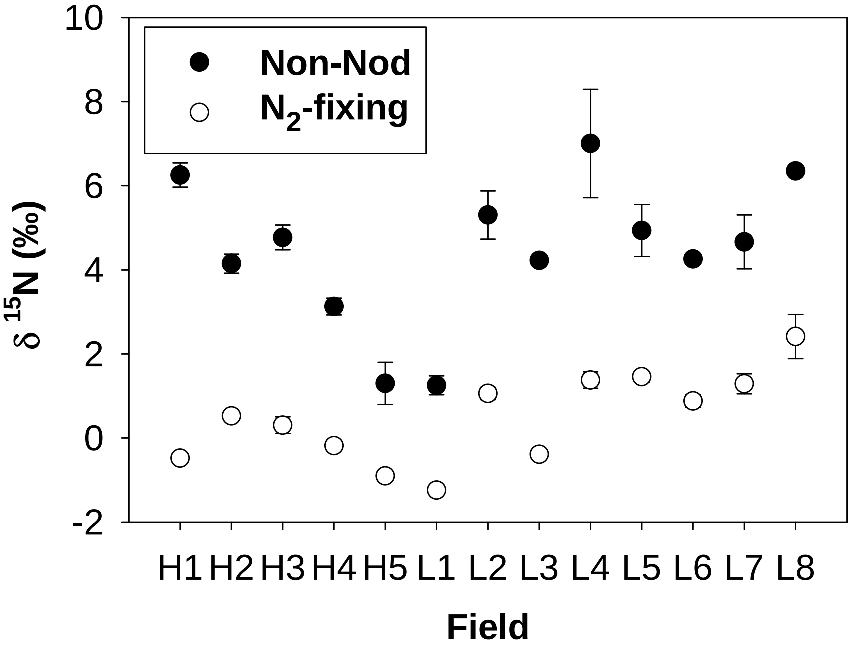


Figure 1.3. Field average $\delta^{15}N$ signatures of N_2-fixing and non-nodulating soybean aboveground biomass at full pod-fill (R6). Error bars represent standard errors (n=4 for non-nod and n=8 for N_2-fixing soybeans).

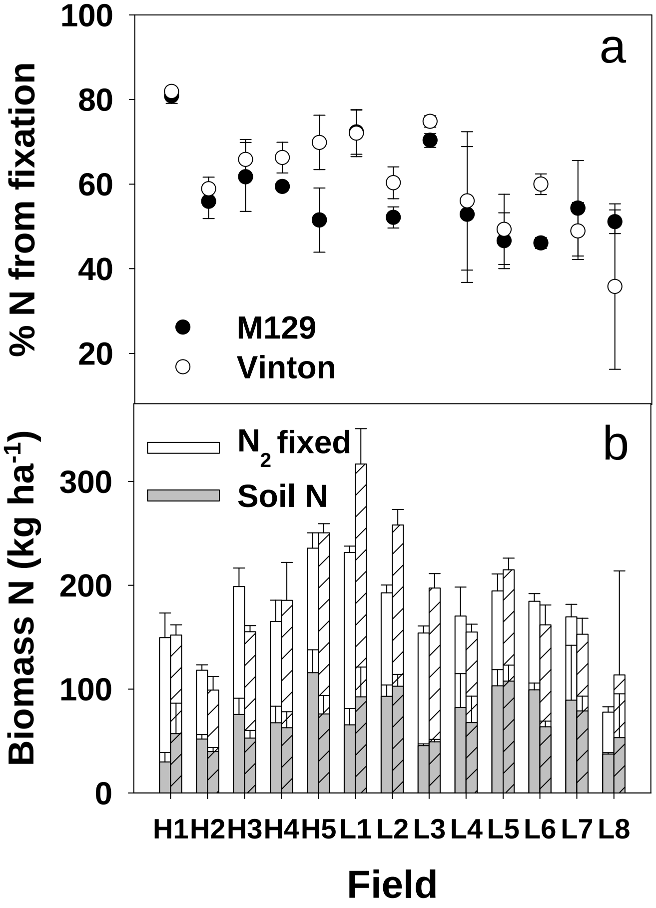


Figure 1.4. Field averages for a) % N from fixation by soybean variety; and b) N_2 fixed and soil N uptake by variety (M129 (open bars) and Vinton 81 (hashed bars)). Error bars represent standard errors (n=4).

Table 1.5. Coefficients of correlation between aboveground biomass of N_2-fixing soybeans, N_2 fixed, % N from fixation, soil N uptake, and non-nodulating soybean biomass N at full pod-fill (R6).

	N_2 fixed	**Soil N uptake**	**% N from fixation**	**Non-nod N[b]**
Biomass	0.89***	0.66*	0.36	0.28
N_2 fixed		0.35	0.61*	0.05
Soil N uptake			-0.38	0.74**
% N from fixation				-0.53

nodulating and non-nodulating soybeans was correlated with several soil N fractions. The ratio of nodulating to non-nodulating soybean soil N uptake increased with decreasing total soil N (Figure 1.5), microbial biomass N (r= -0.69, p=0.009), and oPOM N (r= -0.63, p=0.02).

Analyzing variation in BNF

Aboveground N_2 fixed was strongly correlated with both soybean aboveground biomass production and % N from fixation (Table 1.5). Soil N uptake by the non-nodulating soybeans, a potential indicator of soil N availability, was positively correlated with soil N uptake of the N_2-fixing soybeans, but was not significantly correlated with either total N_2 fixed or % N from fixation (Table 1.5).

The full continuum of soil N pools we measured was negatively correlated with the % N from fixation at R6 with significant correlations for inorganic N and oPOM N (Table 1.6). All of the soil N variables were positively correlated with each other as expected, indicating the strong linkages among soil N pools that play different roles in N cycling and have extremely different turnover times (Table 1.6). The relationship between the % N from fixation and soil N pools did not differ by variety. There were no significant correlations between other soil nutrients (including P, K, Ca, Mg, etc.) and soybean biomass, total N_2 fixed, or % N from fixation (data not shown).

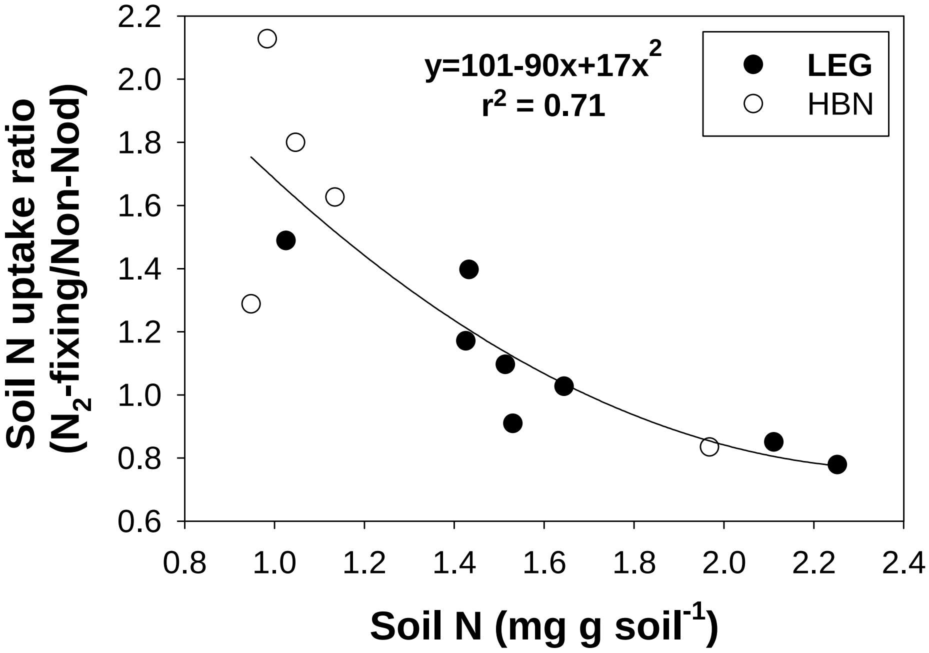


Figure 1.5. Regression between the ratio of N_2-fixing soybean soil N uptake to non-nodulating soybean soil N uptake and total soil N, measured in the top 20 cm, across 13 fields with a history of legume-based N (LEG) and Haber-Bosch N-based (HBN) management.

Table 1.6. Coefficients of correlation between soybean % N from fixation at the final sampling and total soil N (total N), occluded particulate organic matter N (oPOM N), microbial biomass N (MBN), and extractable NO_3^{-1} and NH_4^+ (inorganic N).

	Total N	**log(oPOM N)**	**MBN**	**log(Inorganic N)**
%N from fixation	-0.32	-0.65*	-0.27	-0.68**
Total N		0.62*	0.58*	0.43
Log(oPOM N)			0.40	0.84***
MBN				0.45

* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$

Due to the collinearity of soil variables, we used multivariate analyses to assess the relative importance of soil N pools, other soil nutrients, pH, and soil texture in regulating N_2 fixation and biomass production. We used both principal components analysis (PCA) and forward stepwise regressions to identify which soil variables were the most important in explaining the variation in BNF. PCA resulted in three principal components that accounted for 77% of variation in the soils data (Table 1.7).

Each principal component represents an independent variable that is a composite of several soil variables. PC 1 explained 47% of the variation in the soils data with soil texture (sand and clay) having the strongest, but inverse loadings, followed closely by total soil N, P, MBN and oPOM N (Table 1.7). Total soil N and MBN vectors clustered directionally with soil clay on PC 1 (Figure 1.6). Soil Ca, inorganic N and oPOM N had the strongest loadings on PC 2 which explained 17% of the variation (Table 1.7). Soil P and K had the strongest loadings on PC 3 which explained an additional 13% of the variation.

The PCA results indicated a strong influence of soil texture on the % N from fixation, but did not explain a significant proportion of the variance in total N_2 fixed. The % N from fixation was significantly negatively correlated with PC 1, but not with PC 2 (Table 1.8). The negative correlation with PC 1 indicated decreased reliance on N_2

Table 1.7. Principal Components Analysis eigenvalues and variable loadings for the first 3 principal components explaining a total of 77% of the variation in the data.

	PC1	**PC2**	**PC3**
% variation explained	47%	17%	13%
Eigenvalue	4.19	1.55	1.17
Variable loadings			
Sand	**-0.43**	-0.13	0.19
Clay	**0.41**	0.10	-0.34
P	0.32	0.29	**0.43**
K	-0.16	0.44	**0.61**
Ca	0.26	**0.53**	-0.12
Total soil N	**0.38**	0.05	0.22
MBN[a]	**0.35**	-0.04	-0.16
Log (oPOM N)[b]	0.34	**-0.40**	0.27
Log (Inorganic N)[c]	0.26	**-0.50**	0.36

[a] microbial biomass N; [b] occluded particulate organic matter N; [c] extractable NO_3^{-1} and NH_4^+.

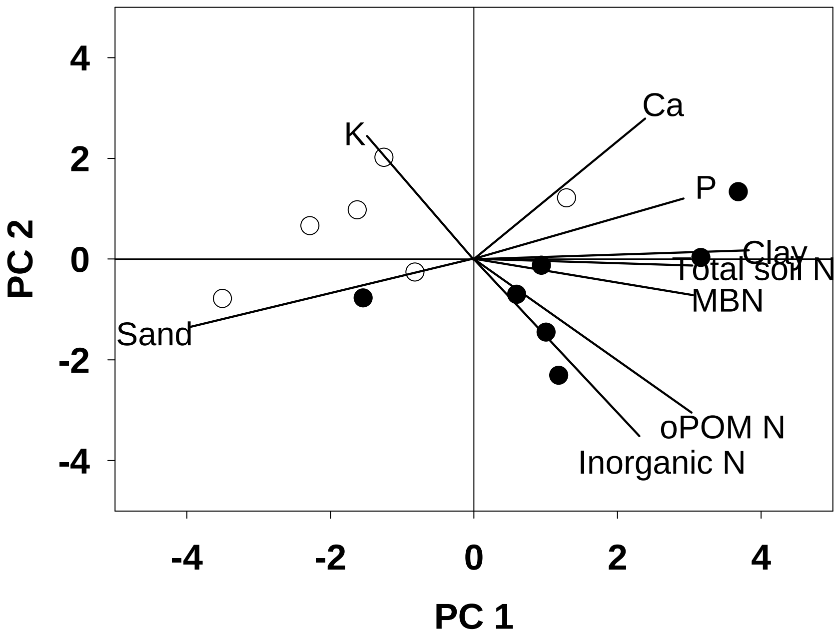


Figure 1.6. Principal components biplot of PC 1 and PC 2. Vectors represent correlations between soil variables and principal components. Open circles represent fields under Haber-Bosch N based management and filled circles represent fields under legume-based management.

fixation with increasing soil clay and total N content. Nitrogen fixation variables were not correlated with PC 2, despite the strong loadings of labile N pools on this component. Aboveground biomass and N_2 fixed were not significantly correlated with any of the principal components (Table 1.8). Fields clustered by management history across PC 1, reflecting the slightly higher average clay content for LEG fields than HBN fields (Figure 1.6). Using covariate analysis, management history did not explain a significant proportion of the variance in the % N for fixation or total N_2 fixed when controlling for soil clay content.

Table 1.8. Coefficients of correlation between plant variables measured at full pod-fill (aboveground biomass of N_2-fixing soybeans (Biomass), N_2 fixed, soil N uptake, % of N from fixation, and non-nodulating biomass N (Non-nod N)), and principal components 1 and 2 (PC 1 and PC 2).

	PC 1	**PC 2**
Biomass	-0.37	-0.33
N_2 fixed	-0.49	-0.19
Soil N uptake	0.12	-0.58*
% N from fixation	-0.65*	0.27
Non-nod N	0.66*	-0.34

* $p<0.05$

The effects of texture and soil N availability on soybean soil N assimilation differed for N_2-fixing and non-nodulating soybeans. The N uptake of the non-nodulating soybeans was positively correlated with PC 1, possibly reflecting the overlap between the N fertility and textural gradients across the fields (Table 1.8). The more labile soil N pools represented by PC 2 were positively correlated with soil N uptake of the N_2-fixing soybeans (Table 1.8).

Stepwise regression results reinforced the importance of soil texture followed by N availability as regulators of N_2 fixation across these fields. Sand concentration and inorganic N explained 20% and 14% of the variation in % N from fixation, respectively (Table 1.9a). The model results did not differ by soybean variety.

Variation in total N_2 fixed was also best explained by a combination of soil texture and soil N variables. Sand concentration and MBN explained a combined 41% of the variability in N_2 fixed and the slopes were positive for both variables (Table 1.9b). Soil sand concentration was the best indicator of site productivity for soybeans across these fields, having the strongest correlation of all soil variables with both soybean biomass and total N_2 fixed ($r = 0.54$, $p = 0.06$; $r = 0.66$, $p = 0.01$, respectively).

Discussion

Variability in soybean nitrogen fixation

While the average proportion of N_2 fixed by soybean plants in this study (60%) is similar to literature values (Unkovich and Pate 2000; Goss et al. 2002), we observed significant variability in both the % N from fixation and total N_2 fixed across these fields. Both biomass production and the relative reliance on N_2 fixation contributed to the nearly 6-fold variation we observed in total N_2 fixed across the 13 fields.

The suppressive effect of soil N availability on BNF was secondary to the environmental and site characteristics that determined the % N from fixation and total N_2 fixed. For example, soil sand content was a strong predictor of % N from fixation (Table 1.9a), suggesting a texture-related effect on the efficiency and effectiveness of the N_2 fixation symbiosis. Soil texture differences also contributed to most of the variation in N_2 fixed with sandier soils supporting greater aboveground biomass and N_2 fixed (Table 1.9b). In contrast, the non-nodulating soybean N uptake was positively correlated with PC 1, reflecting a positive correlation with soil clay content and total soil N (Table 1.8). One of the few other studies that attempted to parse the factors that affect BNF across variable soil types also found higher rates of perennial clover BNF on sandier soils across pastures in Australia (Riffkin et al. 1999). Oxygen availability is an important regulator of nitrogenase activity and legume nodules can have 4 times the oxygen demand of an equal biomass of roots (Layzell and Hunt 1990). Therefore, BNF may be more sensitive to an increased frequency of water saturated conditions in finer textured soils than root assimilation of mineralized soil N in non-fixing plants. Soil textural effects on soil moisture and aeration can also affect plant pathogens. While we did not observe evidence of disease pressure, finer-textured soils can have increased prevalence of soybean stem and root pathogens (Workneh et al. 1999).

Table 1.9. Mixed model results with variables selected using forward stepwise regression that explained a) the variability in the relative reliance of N_2-fixing soybeans on N_2 fixation (% N from fixation) and b) the variability in aboveground N_2 fixed.

a) % N from fixation

$y = 40.4 + 0.80 * \%Sand - 10.2 * \log(Inorganic\ N)$

Variable	df	F	p	% of total variance
Sand	10	8.5	0.02	20%
Log (Inorganic N)	22	3.4	0.08	14%

b) N_2 fixed

$y = -115 - 9 * (M129\ Nod\ Variety) + 4 * \%Sand + 1 * MBN$

Variable	df	F	p	% of total variance
Variety (M129 Nod)	67	10.8	0.002	
Sand	9	2.13	0.001	27%
MBN	35	4.6	0.04	14%

Soil N availability did reduce the % N from fixation, but its impact was secondary to that of soil texture. Soil inorganic N measured at the end of the growing season in a year when no fertilizers were applied represented N that was recently mineralized from endogenous soil N pools. Soil inorganic N concentrations had the strongest negative correlation with % N from fixation (Table 1.6) and, of all of the soil N pools measured, was the best predictor of % N from fixation as selected through stepwise regression (Table 1.9a). The strong positive correlation between inorganic N and oPOM N suggests that oPOM N may be a major source of inorganic N, which, in turn, inhibited BNF (Table 1.6). Occluded POM N is a pool that responds to differences in management legacies over the timeframe of years to decades (Wander et al. 1994; Wander 2004). We found no relationship between soil P or K and soybean growth, suggesting that these nutrients were not limiting in these fields.

Interactions of plant N acquisition strategies and soil nitrogen

While indicators of soil N availability were negatively correlated with the proportion of N from fixation, the response of soil N uptake was more complex. In soils at the more fertile, finer-textured end of the gradient, soil N assimilation by non-nodulating and N_2-fixing soybeans was essentially the same. However, at the lower end of the scale, where reduced soil fertility limited growth of the non-nodulating soybeans, the N_2-fixing soybeans tended to accumulate greater amounts of soil N than the non-nods (Figure 1.5). George et al. (1993) found similar differences across an elevation and fertility gradient with more soil N uptake at low soil N availability in N_2-fixing soybeans compared to the non-nodulating plants. They concluded that these differences indicated a weakness of the ^{15}N natural abundance method. However, these differences in N uptake may not be methodological artifacts, but instead result from a combination of texture- and fertility-related effects. The differences may reflect the ability of the N_2-fixing soybeans to avoid N limitation, increase carbon allocation belowground, and increase access to soil nutrients even as soil reservoirs decline. The increase in belowground carbon allocation could represent a combination of increased root biomass to explore a larger soil volume (Hodge 2003) and increased root C exudation which has been shown to stimulate net N mineralization of SOM pools (Liljeroth et al. 1994; Hamilton and Frank 2001). This differential response of N_2-fixing and non-fixing soybeans to the fertility and textural gradients reveals the complexity of the influence of soil properties on BNF across heterogeneous field sites.

Agroecosystem context

Conducting research in intact agroecosystems provides unique opportunities and challenges. Due to the heterogeneity of farm fields resulting from differences in short- and long-term management histories and soil texture, multicollinear variables make it difficult to conclusively identify causal relationships. However, this heterogeneity allows for analysis of the relative importance of different factors on working farms. For example, we found weak evidence that BNF is regulated by soil N availability from the mineralization of endogenous soil N pools. This contrasts with results from fertilizer-based field studies and hydroponic greenhouse experiments, which have consistently reinforced the concept that soil N availability is the dominant edaphic regulator of BNF (e.g., Hardarson et al. 1984; Streeter 1985; Waterer and Vessey 1993; Goss et al. 2002). Field experiments usually are carried out in the same field and N availability is manipulated across a relatively constant background soil environment (Boller and Nosberger 1994; Goss et al. 2002). We found that N_2 fixation was impacted by both the direct suppressive effect of soil N availability and other soil properties, such as soil texture that may indirectly impact N_2 fixation via oxygen and water saturation dynamics.

The effect of soil N on BNF depends on the relative level of N availability. Fertilizer studies clearly demonstrate that large pools of inorganic N inhibit BNF. The subtle influence of soil N availability on BNF measured in this study suggests that N mineralization from SOM pools represented a much smaller N flux than even the lower levels of N fertilizer used in past studies, which are typically around 50 kg N ha^{-1} (Salvagiotti et al. 2008). The 2-fold range in total soil N across farm fields in this study represents a typical range found across fields in the Northeastern United States. The fields were all productive agricultural fields. In a year when no fertilizers were applied, the N assimilation of non-nodulating soybeans was 28 kg N ha^{-1} in the least fertile field and increased to a maximum of 116 kg N ha^{-1} in the most fertile field. Taken together, our results suggest that soil N availability from SOM mineralization is a relatively minor regulator of soybean BNF on grain farms in this region.

Implications for agroecosystem nitrogen management

Due to the limited number of sites and confounding issues of soil texture in this study, we were not able to confidently distinguish management history from soil type effects on SOM and related soil N pools. The influence of management was significant for only inorganic N availability, oPOM N and N mineralization potential measured at the end of the growing season. Despite a history of surplus fertilizer N additions, the HBN systems had smaller labile N pools and N mineralization potential at the end of the growing season than the LEG fields. Legume-based systems build SOM pools over time (Drinkwater et al. 1998; Gregorich et al. 2001; Marriott and Wander 2006). Mariott and Wander (2006) found that legume-based organic cropping systems had larger POM pools than conventionally managed systems. Puget and Drinkwater (2001) provided an example of one mechanism for improved retention of legume C and N inputs in their finding of increased legume root incorporation into stable soil aggregates in legume-based systems.

Grain legumes vary in their N contributions to agroecosystems depending on the proportion of plant N removed in harvested seed and the proportion of plant N derived from fixation. Seed N exports commonly exceed N_2 fixation inputs for soybean, resulting in a net removal of soil N (Salvagiotti et al. 2008). Nitrogen management recommendations in the Northeast suggest that soybeans provide a small N benefit in the range of 20-30 kg N ha^{-1} for succeeding crops, which reflects N_2 fixation inputs, effects of soybean above- and belowground residue mineralization, and the indirect stimulation of SOM decomposition by low C:N soybean residue inputs (Ketterings et al. 2007). Because we harvested soybeans prior to leaf senescence to measure total NPP and due to plot size limitations, we did not directly measure seed yields. However, we can estimate soybean net N balances using our NPP measurements because harvest index (ratio of seed yield to total aboveground plant biomass) is typically stable within a given variety (Spaeth et al. 1984).

Using measurements of N_2 fixation and shoot N, and estimates of harvest index (HI), seed N concentration, and belowground plant N, we estimated the annual net soybean N balance for Vinton 81 soybeans in each field. We estimated an HI of 0.36, based on measurements from farms included in this study of plant biomass for Vinton 81 soybeans at peak growth and seed yields at full maturity during previous experiments (unpublished data). This value is in agreement with the average HI value measured by Johnson and Major (1979) across Maturity Group I soybean varieties. We assumed an average seed N concentration of 6.8% N, based on average values measured for Vinton 81 soybean seed N concentration across 15 fields in the study area in 2002, 2003, and 2004 (unpublished data). To estimate belowground N, we assumed that 24% of total plant N was in belowground biomass at soybean maturity, based on data from Rochester et al. (1998). Field average soybean N inputs were estimated using our measurements of aboveground soybean biomass multiplied by 1.24 to include root N and then multiplied by our field measurements of % N from fixation.

We estimate that N balances for Vinton 81 soybeans varied more than 5-fold across the 13 farm fields, from -16 to +73 kg N ha^{-1}, and averaged +20 kg N ha^{-1} (Figure 1.7). The % N from fixation is a stronger predictor of soybean N balances than total biomass accumulation due to the constant ratio between biomass accumulation and seed N removal (Peoples and Craswell 1992). For the Vinton 81 soybeans, positive balances occurred when the % N from fixation was greater than 60%, the average measured in this study (Figure 1.7).

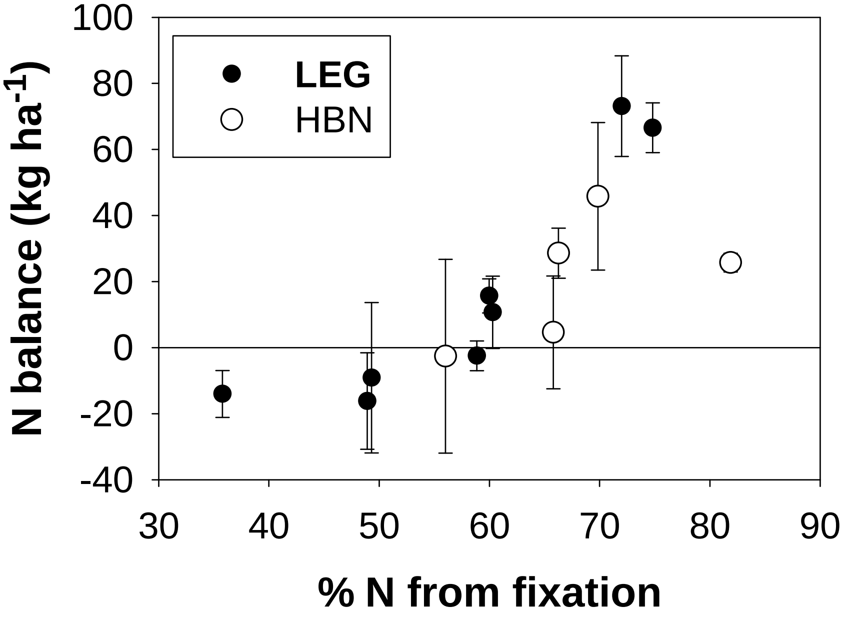


Figure 1.7. Relationship between Vinton 81 soybean % N from fixation and estimated annual soybean N balance for fields with a history of legume-based N (LEG) and Haber-Bosch N-based (HBN) management. Error bars represent standard errors (n=4).

The relatively small influence of soil N availability on N_2 fixation and, therefore, soybean N balance, suggests that soybeans do not serve as internal regulators of N cycling dynamics in these agroecosystems. Soybeans have undergone intensive breeding selection, often under high soil N conditions, which has altered their interactions with rhizobial symbionts. For example, Kiers et al. (2007) found that newer soybean varieties were less effective at sanctioning ineffective rhizobial symbionts than older varieties. These changes and other modifications may have impaired their ability to effectively down-regulate N_2 fixation in the presence of soil N. Non-grain, perennial and winter annual legumes, which have undergone less intensive breeding selection, typically provide the main source of N inputs within most legume-based systems. For example, the legume-based farms in this study integrated legumes into diversified cash-grain rotations at intervals that balanced crop N exports primarily through the use of red clover (*Trifolium pratense*) interseeded into winter grain fields. Further research is required to understand whether BNF of legume species used commonly as green manures respond to edaphic factors similarly to soybean, when used within diversified, temperate cropping systems.

Conclusion

Soil N availability is often considered to be a central factor affecting BNF. We found that the suppressive effects of soil N availability on BNF were secondary to site characteristics, such as soil texture and corresponding soil characteristics that determined total NPP. BNF interacted with the fertility gradient and conferred an advantage to accessing soil N in low fertility sites through increased C fixation of N_2-fixing plants relative to non-fixing plants. We found weak evidence of management history influence on either soil N pools or N_2 fixation due to soil textural differences across the sites.

This study contributes to our understanding of the ecology of legume N_2 fixation in agroecosystems and the interactions of plant N acquisition strategies and SOM dynamics. It illustrates the importance of testing relationships indentified under relatively controlled experimental conditions within the context of heterogeneous agroecosystems to understand their functional significance. This study also provides a starting point for future research to develop decision-support tools for farmers that provide field-specific estimates of BNF to improve nutrient management practices. Further research is necessary to understand how to manage legume BNF in legume-based systems across a range of soil types to improve N cycling efficiency and maintain crop productivity.

REFERENCES

Blumenthal, J.M., M.P. Russelle, and C.P. Vance (1997) Localized and internal effect of nitrate on symbiotic dinitrogen fixation. Physiologia Plantarum 101:59-66

Boller BC, Nosberger J (1994) Differences in nitrogen-fixation among field-grown red clover strains at different levels of ^{15}N fertilization. Euphytica 78:167-174

Bonde TA, Rosswall T (1987) Seasonal variation of potentially mineralizable nitrogen in 4 cropping systems. Soil Science Society of America Journal 51:1508-1514

Boone RD (1994) Light-fraction soil organic matter: origin and contribution to net nitrogen mineralization. Soil Biology & Biochemistry 26:1459-1468

Clarholm M (1985) Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. Soil Biology & Biochemistry 17:181-187

Corre MD, Schnabel RR, Stout WL (2002) Spatial and seasonal variation of gross nitrogen transformations and microbial biomass in a Northeastern US grassland. Soil Biology & Biochemistry 34:445-457

Drinkwater LE, Cambardella CA, Rice CW (1996) Potentially mineralizable N as an indicator of active soil N. In: Doran J, Jones AJ (eds) Methods for assessing soil quality. Soil Science Society of America, Madison, WI

Drinkwater LE, Snapp SS (2007) Nutrients in agroecosystems: rethinking the management paradigm. Advances in Agronomy 92:163-186

Drinkwater LE, Wagoner P, Sarrantonio M (1998) Legume-based cropping systems have reduced carbon and nitrogen losses. Nature 396:262-265

Fehr WR, Caviness CE, Burmood DT, Pennington JS (1971) Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. Crop Science 11:929-931

Freund, R.J., and R.C. Littell (2000) SAS System for regression. SAS Publishing, Gary, NC

Gale WJ, Cambardella CA, Bailey TB (2000) Root-derived carbon and the formation and stabilization of aggregates. Soil Science Society of America Journal 64:201-207

Galloway JN, Aber JD, Erisman JW, Seitzinger SP, Howarth RW, Cowling EB, Cosby BJ (2003) The nitrogen cascade. Bioscience 53:341-356

Galloway JN, Cowling EB (2002) Reactive nitrogen and the world: 200 years of change. Ambio 31:64-71

Gan YB, Stulen I, van Keulen H, Kuiper PJC (2004) Low concentrations of nitrate and ammonium stimulate nodulation and N_2 fixation while inhibiting specific nodulation (nodule DW g^{-1} root dry weight) and specific N_2 fixation (N_2 fixed g^{-1} root dry weight) in soybean. Plant and Soil 258:281-292

George T, Singleton PW, van Kessel C (1993) The use of ^{15}N natural abundance and nitrogen yield of non-nodulating isolines to estimate nitrogen fixation by soybeans (*Glycine max* L.) across three elevations. Biology and Fertility of Soils 15:81-86

Goss MJ, de Varennes A, Smith PS, Ferguson JA (2002) N_2 fixation by soybeans grown with different levels of mineral nitrogen, and the fertilizer replacement value for a following crop. Canadian Journal of Soil Science 82:139-145

Gregorich EG, Drury CF, Baldock JA (2001) Changes in soil carbon under long-term maize in monoculture and legume-based rotation. Canadian Journal of Soil Science 81:21-31

Hamilton EW, III, Frank DA (2001) Can plants stimulate soil microbes and their own nutrient supply? Evidence from a grazing tolerant grass. Ecology 82:2397-2402

Hardarson, G., F. Zapata, and S.K.A. Danso (1984) Effect of plant genotype and nitrogen-fertilizer on symbiotic nitrogen-fixation by soybean cultivars. Plant and Soil 82:397-405

Hodge A (2003) Plant nitrogen capture from organic matter as affected by spatial dispersion, interspecific competition and mycorrhizal colonization. New Phytologist 157:303-314

Howarth RW, Paul EA (1994) Microbial biomass. In: Weaver RW, Angle JS, Bottomley P (eds) Methods of soil analysis, Part 2. Microbial and biochemical properties. Soil Science Society of America, Madison, WI, pp 753-773

Johnson, D.R., and D.J. Major. 1979. Harvest index of soybeans as affected by planting date and maturity rating. Agronomy Journal 71:538-541.

Jones DL, Shannon D, Murphy DV, Farrar J (2004) Role of dissolved organic nitrogen (DON) in soil N cycling in grassland soils. Soil Biology & Biochemistry 36:749-756

Ketterings, Q., S. Swink, B. Cox, and K. Czymmek. 2007. Soybean N credits Fact Sheet 30. Cornell University Cooperative Extension.

Kiers ET, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume-rhizobium mutualism. Nature 425:78-81

Layzell DB, Hunt S (1990) Oxygen and the regulation of nitrogen fixation in legume nodules. Physiologia Plantarum 80:322-327

Liljeroth E, Kuikman P, Van Veen JA (1994) Carbon translocation to the rhizosphere of maize and wheat and influence on the turnover of native soil organic matter at different soil nitrogen levels. Plant and Soil 161:233-240

Marriott EE, Wander MM (2006) Total and labile soil organic matter in organic and conventional farming systems. Soil Science Society of America Journal 70:950-959

McGill WB, Cannon KR, Robertson JA, Cook FD (1986) Dynamics of soil microbial biomass and water-soluble organic C in Breton L after 50 years of cropping to 2 rotations. Canadian Journal of Soil Science 66:1-19

McIsaac GF, David MB, Gertner GZ, Goolsby DA (2001) Eutrophication - Nitrate flux in the Mississippi River. Nature 414:166-167

Midwood AJ, Boutton TW (1998) Soil carbonate decomposition by acid has little effect on delta ^{13}C of organic matter. Soil Biology & Biochemistry 30:1301-1307

O'Rourke N, Hatcher L, Stepanski EJ (2005) A step-by-step approach to using SAS for univariate and multivariate statistics, 2nd Ed. John Wiley, New York

Peoples, M.B., and E.T. Craswell. 1992. Biological nitrogen-fixation - Investments, expectations and actual contributions to agriculture. Plant and Soil 141:13-39.

Puget P, Drinkwater LE (2001) Short-term dynamics of root- and shoot-derived carbon from a leguminous green manure. Soil Science Society of America Journal 65:771-779

Riffkin PA, Quigley PE, Kearney GA, Cameron FJ, Gault RR, Peoples MB, Thies JE (1999) Factors associated with biological nitrogen fixation in dairy pastures in south-western Victoria. Australian Journal of Agricultural Research 50:261-272

Rochester IJ, Peoples MB, Constable GA, Gault RR (1998) Faba beans and other legumes add nitrogen to irrigated cotton cropping systems. Australian Journal of Experimental Agriculture 38:253-260

Ross DJ (1987) Soil microbial biomass estimated by the fumigation incubation procedure - Seasonal fluctuations and influence of soil-moisture content. Soil Biology & Biochemistry 19:397-404

Ross, S.M., R.C. Izaurralde, H.H. Janzen, J.A. Robertson, and W.B. McGill. (2008) The nitrogen balance of three long-term agroecosystems on a boreal soil in western Canada. Agriculture Ecosystems & Environment 127:241-250

Salvagiotti F, Cassman KG, Specht JE, Walters DT, Weiss A, Dobermann A (2008) Nitrogen uptake, fixation and response to fertilizer N in soybeans: A review. Field Crops Research 108:1-13

Schmidt SK, Costello EK, Nemergut DR, Cleveland CC, Reed SC, Weintraub MN, Meyer AF, Martin AM (2007) Biogeochemical consequences of rapid microbial turnover and seasonal succession in soil. Ecology 88:1379-1385

Shearer G, Kohl DH (1986) N_2 fixation in field settings: estimates based on natural ^{15}N abundance. Australian Journal of Plant Physiology 13:699-756

Snijders TAB, Bosker RJ (1999) Multilevel analysis: an introduction to basic and advanced multilevel modeling. SAGE Publications, London

Spaeth, S.C., H.C. Randall, T.R. Sinclair, and J.S. Vendeland. 1984. Stability of soybean harvest index. Agronomy Journal 76:482-486.

Stevenson FJ (1994) Humus chemistry: Genesis, composition, reactions. John Wiley and Sons, New York

Streeter JG (1985) Nitrate inhibition of legume nodule growth and activity. 2. Short-term studies with high nitrate supply. Plant Physiology 77:325-328

Unkovich MJ, Pate JS (2000) An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes. Field Crops Research 65:211-228

Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm NB, Howarth RW, Marino R, Martinelli L, Rastetter EB, Sprent JI (2002a) Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 57/58:1-45

Vitousek PM, Hattenschwiler S, Olander L, Allison S (2002b) Nitrogen and nature. Ambio 31:97-101

von Lutzowa M, Kogel-Knabner I, Ekschmittb K, Flessa H, Guggenberger G, Matzner E, Marschner B (2007) SOM fractionation methods: Relevance to functional pools and to stabilization mechanisms. Soil Biology & Biochemistry 39:2183-2207

Wander M (2004) Soil organic matter fractions and their relevance to soil function. In: Magdoff F, Weil RR (eds) Soil organic matter in sustainable agriculture. CRC Press, Boca Raton, FL

Wander MM, Traina SJ, Stinner BR, Peters SE (1994) The effects of organic and conventional management on biologically-active soil organic matter pools. Soil Science Society of America Journal 58:1130-1139

Waterer JG, Vessey JK (1993) Effect of low static nitrate concentrations on mineral nitrogen uptake, nodulation, and nitrogen fixation in field pea. Journal of Plant Nutrition 16:1775-1789

Woodmansee R (1984) Comparative nutrient cycles of natural and agricultural ecosystems: a step toward principles. In: Lowrance BR, Stinner R, House GJ (eds) Agricultural ecosystems: unifying concepts. J. Wiley, New York, pp 145-156

Workneh E, Yang XB, Tylka GL (1999) Soybean brown stem rot, *Phytophthora sojae*, and *Heterodera glycines* affected by soil texture and tillage relations. Phytopathology 89:844-850

Zapata F, Danso SKA, Hardarson G, Fried M (1987) Time course of nitrogen fixation in field-grown soybean using ^{15}N methodology. Agronomy Journal 79:172-176

CHAPTER 2

THE ECOLOGY OF RED CLOVER NITROGEN FIXATION INTERSEEDED WITH WINTER CEREAL GRAINS

Abstract

Incorporating legume cover crops into annual grain agroecosystems can reduce the negative environmental impacts of farming systems and improving our understanding of the ecology of nitrogen (N) fixation in agroecosystems can reduce farmers' risks of relying on variable legume N inputs. Using the ^{15}N natural abundance method, we estimated the N fixation of red clover (*Trifolium pratense*) interseeded into winter cereal grains, a common farmer practice, across a management-induced fertility gradient. Farm fields were classified as being under either legume-based (LEG) or Haber Bosch N fertilizer (HBN)-based management practices using the relative contributions of legume N fixation and HBN to total N inputs. To understand the interactive effects of grains on legume N fixation, we compared interseeded clover to monoculture clover and clover-orchardgrass (*Dactylis glomerata*) mixtures. The LEG fields had greater quantity and quality of soil organic matter and lower P and K availability than HBN fields. Particulate organic matter (POM) N and POM C:N were greater in LEG than HBN fields. Orchardgrass biomass was positively correlated with the management-induced N fertility gradient, but we found no evidence of soil N availability suppressing BNF. The average % N from fixation in interseeded clover was 73% in the fall and 65% the following spring, which was similar to the % N from fixation in clover-grass mixtures. Total N fixed in interseeded clover aboveground biomass ranged from 23 to 87 kg N ha^{-1} at the end of the first growing season and the average was similar to clover monocultures. Interseeding clover with grain improved clover N fixation in comparison to a red

clover monoculture without affecting the grain crop. Grain type exerted a strong top-down effect on clover biomass likely due to competition for light between clover and taller spelt varieties.

Introduction

Legume cover crops can serve multiple functions in agroecosystems, including weed suppression, soil erosion reduction, nutrient retention, and increased nitrogen (N) availability from biological N fixation (BNF). Incorporation of legume cover crops into annual grain rotations remains limited, despite extensive evidence that they can reduce negative environmental impacts of agroecosystems while maintaining crop yields (Lotter et al. 2003; Snapp et al. 2005; Tonitto et al. 2006). Two primary constraints to the further reliance on legume cover crops are the limited niches available for including cover crops without foregoing a crop harvest every year and the uncertainty of the actual N inputs from legume cover crops.

Diversified grain rotations that include a winter cereal grain harvested mid-summer have a unique niche for the inclusion of cover crops. Interseeding perennial red clover (*Trifolium pratense*) into winter grains is a common practice in some regions of North America. Red clover is typically frost-seeded into a winter grain and then allowed to grow as a monoculture following grain harvest. Interseeded red clover can provide sufficient N for a succeeding corn (*Zea mays*) crop in addition to suppressing weeds in the winter grain and providing soil cover if allowed to grow through the winter (Bruulsema and Christie 1987; Vyn et al. 1999). Because only some of the legume N will become available to the following crop during the first year following incorporation, research on red clover N contributions have focused on measuring the N fertilizer replacement value rather than measurement of the biomass N inputs (Bruulsema and Christie 1987). No published studies have measured N fixation inputs in these systems.

Understanding legume N fixation inputs is critical for N mass balance calculations. Nutrient mass balances are useful performance-based metrics that can aid in nutrient management decision-making (Watson et al. 2002). Large positive balances indicate the potential for management practices to result in nutrient losses to the surrounding environment and negative balances indicate the potential for the degradation of soil pools over time. Multi-year nutrient mass balances are a particularly useful management tool for diversified, legume-based systems because legume N inputs cycle through SOM pools and become available over several years. Single year measurements of crop responses following legume incorporation do not capture these longer-term dynamics. Mass balances are also an important tool for understanding the ecology of agroecosystems. For example, connecting longer-term mass balance data with soils data can reveal the relative efficiency of nutrient cycling and retention in legume-based systems relative to conventionally fertilized systems (Drinkwater et al. 1998; Ross et al. 2008).

Legume N inputs are a source of uncertainty in constructing mass balances due to the high spatial and temporal variability of BNF (Carlsson and Huss-Danell 2003). Soil N availability may be one important driver of N fixation in agroecosystems. Due to the high energetic costs of N fixation to the host plant, legumes preferentially take up soil N when available and reduce their reliance on N fixation. The increased soil N availability measured in legume-based systems could regulate N fixation inputs by inhibiting N fixation rates. However, the ability of endogenous soil N reservoirs, and internal N cycling processes such as mineralization, to regulate BNF has been studied only rarely because inorganic N additions typically are used to produce N-fertility gradients (e.g., Elgersma et al. 2000; Gan et al. 2002; Goss et al. 2002).

In addition to the potential for bottom-up effects of soil fertility to influence N fixation of red clover interseeded with winter grains, top-down competitive effects of the grain crop can influence clover growth and N fixation. Earlier studies of interseeded clover-winter grain systems identified light competition as a factor influencing clover growth, and shorter-statured spring grains were recommended for the establishment of perennial legume-based forages (Bula et al. 1954). Winter wheat (*Triticum aestivum* L. subsp. *aestivum*)-clover intercrops have been increasingly recommended because modern wheat cultivars are now similar in stature to spring grains (Blaser et al. 2006). Crop rotations on organic grain farms in the Northeast commonly include spelt (*Triticum aestivum* L. subsp. *spelta*), a winter grain that has undergone less intensive breeding selection than modern wheat varieties and is similar in stature to taller, older varieties of wheat. Spelt also has a later harvest date than wheat, extending the overlap period of clover and grain. There have been no published studies of the effects of spelt production on interseeded clover growth and N fixation.

Intercropping species in agroecosystems can confer several advantages over monocultures, including increased land equivalent production due to complementary resource use (Vandermeer 1989). However, competitive interactions between species for resources such as light availability can overwhelm facilitative interactions. Relay intercrops, in which the period of interspecies competition is limited, can have higher productivity in comparison to simultaneous intercrops because each crop has a compensatory monoculture growth period (Zhang and Li 2003).

To study the ecology of nitrogen fixation in red clover-winter grain intercrops, we established research plots across 15 farm fields in central New York. Our primary objective was to investigate how management-driven soil fertility differences influenced N fixation of red clover interseeded with a winter grain. In particular, we asked whether farm fields under legume-based management practices differed in soil organic matter quantity and quality compared with fields under HBN-based management practices and how these differences influenced N fixation. To understand the relative effects of the grain-clover relay intercrop on clover growth and N fixation, we compared the interseeded clover-grain management practice to red clover grown in monoculture and in mixture with a grass.

Materials and Methods

Experimental sites

Research plots were established in 2006 in 15 fields on seven commercial grain farms in central New York state (42° 36'-42° 44' N and 77° 03'-76° 42' W). All fields were located within 30 km of each other. The climate is characterized as humid temperate with a mean annual precipitation of 880mm and mean annual maximum and minimum air temperature of 14°C and 3°C, respectively. Weather data from the Penn Yan Airport, Penn Yan, NY (within 10 km SW of fields L2, L3, L4, H2, H5, H6, and M1), and the Aurora Research Farm, Aurora, NY (within 15 km NE of the remaining fields) were averaged for data covering the study period and the 30-year historical average (National Climate Data Center).

Fields were selected to reflect a gradient of management histories across soils with similar texture (Table 2.1). All soils are moderately well to well drained, mixed, active, mesic, Hapludalfs (USDA-NRCS SSURGO database). All fields had similarly diverse rotations including corn, annual grain legumes, and winter cereal grains. Three fields had no history of legume cover crop use and the other fields ranged from one to 17 years of legume cover crop use. To expand the gradient to include fields that have been under longer-term legume-based management, fields managed using organic practices were included. Organic growers in the region primarily grow spelt rather than wheat. Therefore, four of the fields in the study were in spelt, one field was

Table 2.1. Field management history, including years under organic management, % of N inputs from legume BNF over 5-year rotation cycle, crop rotation, and soil type.

Field[a]	Years Org	% N inputs from BNF	5-year rotation[b]	Soil classification
H1	0	8	corn-corn-corn-kidney beans-wheat/clover	Fine sandy-loam, Oxyaquic Hapludalf
H2	0	34	corn-soybean-corn-snap bean-wheat	Silt loam, Oxyaquic Hapludalf
H3	0	49	snap beans-wheat/clover-corn-snap beans-wheat/clover	Fine sandy-loam, Oxyaquic Hapludalf
H4	0	33	soybean-corn-corn-soybean-wheat	Silt loam, Oxyaquic Hapludalf
H5	0	49	snap beans-wheat/clover-corn-snap beans-wheat/clover	Fine sandy-loam, Oxyaquic Hapludalf
H6	0	16	hay-corn-corn-snap beans-wheat	Silt loam, Oxyaquic Hapludalf
M1	0	60	soybean-wheat/clover-corn-soybean-wheat/clover	Gravelly loam, Glossic Hapludalf
M2	16	46	soybean-wheat/clover-corn-kidney bean-spelt/clover	Fine silt loam, Glossic Hapludalf
M3	6	42	fallow-clover-corn-fallow-wheat/clover	Silt loam, Oxyaquic Hapludalf
L1	3	62	hay-hay-soybean-fallow-spelt/clover	Silt loam, Oxyaquic Hapludalf
L2	12	65	soybean-spelt/clover-corn-oats/peas-barley/clover	Fine silt loam, Glossic Hapludalf
L3	16	57	cabbage-spelt/clover-corn-kidney bean-wheat/clover	Fine silt loam, Glossic Hapludalf
L4	7	88	spelt/clover-cabbage-clover-clover-spelt/clover	Fine sandy-loam, Glossic Hapludalf
L5	17	60	soybean-wheat/clover-corn-soybeans-wheat/clover	Silt loam, Oxyaquic Hapludalf
L6	7	68	hay-soybean-wheat/clover-snap beans-spelt/clover	Gravelly silt loam, Glossaquic Hapludalf

[a] H=Haber-Bosch fertilizer N-based management; M=mixed HBN-legume or legume-manure management; L=legume-based management

[b] Cropping years are separated by dashes and intercroppings are separated by slashes with 2006 crop on right. Corn (*Zea mays*); kidney bean and snap bean (*Phaseolus vulgaris*); rye (*Secale cereale*); wheat (*Triticum aestivum*); spelt (*Triticum spelta*); clover (*Trifolium pratense*); oats (*Avena sativa*); peas (*Pisum sativum*); cabbage (*Brassica oleracea*)

planted to winter barley (*Hordeum vulgare*), and the remaining 10 fields were planted to different varieties of winter wheat.

A 5-year cropping history was compiled from participating farmers including all N inputs and crop yields for each field. Published values and data from several years of compost, manure, cover crop, and grain N sampling and analysis in the region were combined with farmer records of field inputs and exports to calculate estimated N inputs for each field. Nitrogen fixation inputs were the greatest source of uncertainty in N inputs. To estimate BNF inputs, we used the following equations:

$$\text{Red clover BNF} = \text{biomass} * (\text{biomass } \%\text{N}/100) * \%\ \text{Nfix} * (1+\text{root N}) \quad (1)$$

$$\text{Grain legume BNF} = (\text{Grain yield}/\text{HI}) * (\text{biomass } \%\text{N}/100) * \%\ \text{Nfix} * (1+\text{root N}) \quad (2)$$

Harvest index (HI) is the ratio of seed yield to total plant biomass. Values used for each legume are presented in Table 2.2.

Table 2.2. Values for biomass N concentration, harvest index, the % N from fixation (% Nfix), and root N (proportion of total plant N in roots) used in equations 1 and 2 to estimate legume N fixation inputs over a 5-year crop rotation for each field.

Crop	Biomass % N	Harvest Index	% Nfix	Root N
Red clover	3.5	NA	65	0.43[a]
Snap beans	2.5	0.50[b]	50[c]	0.18[d]
Kidney beans	2.5	0.50[b]	50[c]	0.18[d]
Soybeans	2.5	0.36[e]	60[e]	0.24[f]
Field pea	3.2	0.50[g]	80 [h]	0.15[i]

[a] Hogh-Jensen et al. 2004 ; [b] Bliss 1993; [c] Hardarson et al. 1993; [d] Fageria and Santos 2008; [e] See Chapter 1 ; [f] Rochester et al. 1998 ; [g] Lecouer and Sinclair 2001; [h] Hauggard-Nielsen et al. 2008; [i] Mayer et al. 2003

The 5-year management histories were used to classify each field based on the percent of total N inputs derived from either HBN fertilizers or legume BNF inputs.

The fields under primarily legume-based management relied on BNF for the majority of total N inputs. Nitrogen management categories were defined as: 1) >50% N inputs from HBN (HBN); and 2) >50% N inputs from legume BNF (LEG). All LEG fields were certified organic, ranging from 3 to 17 years since conversion to organic management practices (Table 2.1). Three fields did not fit into either management category because they either used HBN, but relied on BNF for more than 50% of N inputs (Field M1), or did not use HBN, but relied on BNF for less than 50% of N inputs with the remainder of N inputs from manure inputs (Fields M2 and M3). These three fields were grouped into a mixed category (MIX).

Plot establishment and management

Plots were established in a split-plot design with four replicate blocks per field. All collaborating farmers used conventional tillage practices and had seeded fields in the study to spelt, barley or wheat in the fall of 2005 using 19cm row spacing. The 'medium' red clover variety was used in all fields. Paired plots of 1.5-m by 9.1-m were established with and without red clover in each block. For all LEG fields, except field L4, and fields H2, H5, and M1, farmers broadcast red clover seed between February 22 and April 21 at the rate of 11.2 kg/ha. To maintain grain plots without clover, we covered plots in each block with row cover during farmer seeding. In the remaining fields, we hand broadcast red clover seed into winter grain strips at the same rate of 11.2 kg/ha between March 8 and April 21.

Winter grains were removed from a separate set of adjacent plots within each block to establish red clover monocultures, clover-orchardgrass (*Dactylis glomerata*) mixtures, and monoculture orchardgrass to serve as a reference plant for N fixation estimates. Within each block, spring winter grain growth was removed from a 1.5-m by 9.1-m area using flame weeding in LEG fields and field M1, or glyphosate treatment in HBN fields and fields M2 and M3, in March and April 2006. We removed senescent aboveground biomass from herbicide-treated fields to produce similar starting conditions in all plots. Into cleared subplots measuring 1.5-m by 3.0-m, we hand-broadcast perennial red clover and orchardgrass seed from May 5-7, 2006 at the rates of 35 kg/ha for monoculture red clover (RC), 30 kg/ha for monoculture orchardgrass (OG), and 20 kg/ha for red clover and 15 kg/ha for orchardgrass in mixed plots (RC/OG).

Farmers applied no fertilizers or amendments to the plots during the experiment with two exceptions. Composted poultry manure was spread on L2 at the rate of 3.4 t/ha and composted dairy manure was spread on H4 at an unknown rate in October 2006. Weeds were controlled by hand-weeding. Field M3 was inadvertently plowed after sampling in 2006 and, therefore, no spring data were collected from this field.

Grain harvest and analysis

Grains were harvested by hand from 0.25 m^2 quadrats and straw height was measured within each plot between July 3 and July 17. Grain was dried, threshed, weighed, and ground. Grain samples were analyzed for total N and C using a LECO CN-2000 (Leco Corporation, St. Joseph, MO). Farmers harvested grain straw from some of the fields. Estimates of straw yields were supplied by farmers and N removed in straw was calculated using straw nutrient content data from the USDA NRCS Plant Nutrient Content Database.

Plant sampling and analysis

We sampled 0.25 m^2 of aboveground biomass from all subplots, avoiding plot edges, between October 8 and October 26 and then again between April 24 and May 7, 2007. If weeds were present, weed weights were measured. Weed biomass never amounted to more than 9% of total biomass and was not included in biomass estimates for analysis. Biomass was dried at 60° C and first coarsely ground using both a hammer mill and grinder and then finely pulverized using a roller grinder. Samples were analyzed for ^{15}N natural abundance and total N content using a continuous flow Isotope Ratio Mass Spectrometer (Stable Isotope Facility, UC Davis).

Nitrogen fixation

Nitrogen fixation was calculated using the ^{15}N natural abundance method (Shearer and Kohl 1986). The proportion of N derived from atmosphere in legume biomass (% N from fixation) was calculated using the orchardgrass as the reference plant for the red clover:

$$\%\text{ N from fixation} = 100*((\delta^{15}\text{N Grass} - \delta^{15}\text{N legume})/(\delta^{15}\text{N Grass} - B)) \qquad (3)$$

where B is the $\delta^{15}N$ value of the legume grown with atmospheric N_2 as the only source of N after accounting for seed N. Total N fixed was calculated from the aboveground dry matter of the legume and its biomass N concentration and % N from fixation.

To quantify the B value, we grew red clover in N-free, autoclaved, calcined clay media (Turface©, AIMCOR, Deerfield, IL) in a greenhouse. We surface sterilized seeds in 70% (v/v) ethanol for 3 minutes and 3% (v/v) bleach solution for 2 minutes, followed by a 3 minute rinse in deionized water. We applied the same inoculant as used in field plots to sterilized seeds and fertilized plants with an N-free Hoagland's nutrient solution (GreenCare Fertilizers, Chicago, IL). Plants were sampled at 17 weeks after planting and were processed using the same methods as plant samples from field plots. The resulting B value used for N fixation calculations was -1.65.

Description of soil N fractions measured

A range of soil N fractions were quantified to span the continuum from labile to more recalcitrant N pools based on their availability for microbial mineralization. The different soil N fractions measured interact with environmental factors to determine soil N availability. Inorganic N is an ephemeral soil N pool with high temporal and spatial variability. Inorganic N pool size depends on inputs from microbially-mediated mineralization and losses from denitrification, volatilization, leaching, and plant and microbial immobilization. Microbial biomass (MB) is sensitive to temperature and moisture availability and represents an important sink, as well as a source, for inorganic N depending on C availability. Organic matter can be further partitioned into physical fractions to separate more labile pools from older, recalcitrant pools. Light fraction particulate organic matter (fPOM), also referred to as free POM because it is not associated with soil aggregates, is derived from recent litter inputs and can represent a sink or a source for inorganic N, depending on the source material C:N ratio and biochemistry (Boone 1994; Wander 2004). Occluded particulate organic matter (oPOM), is physically protected within soil aggregates and is more likely to be a net source of inorganic N than fPOM because it typically is more decomposed, resulting in a lower C:N ratio (Wander 2004; Marriott and Wander 2006).

Soil sampling and analysis

We collected soil samples at three time points during 2006. Between June 7 and June 15, we sampled soils across each replicate block (Sampling 1). Eighteen soil cores (2-cm diameter by 20-cm depth) were taken and composited from each block within each field. Between August 9 and August 11, we sampled soils following winter grain harvest (Sampling 2). Six soil cores (2-cm diameter by 20-cm depth) were taken and composited from each block within each field. In October, we sampled soils from each individual subplot at the time of fall biomass sampling (Sampling 3). We used 7-cm Dutch augers to collect approximately 2 kg of soil from each plot to 20-cm depth. To measure soil bulk density, we collected four soil cores of exactly 2-cm diameter by 20-cm depth from 8 randomly selected plots within each field. Table 2.3 outlines the analyses completed for the different sampling times.

Table 2.3. Soil analyses completed for different soil sampling points during 2006.

Sampling	Month	Soil analyses
1	June	Inorganic N[a], N min[b], texture, Morgan-extractable nutrients[c], total C and N
2	August	Inorganic N, N min
3	October	Inorganic N, N min, DOC[d], DON[d], MB[e], bulk density, POM[f]

[a] Extractable NO_3^- and NH_4^+
[b] N mineralization potential
[c] Morgan-extractable P, K, Ca, Mg, Cu, Zn, Fe, Al, and Mn
[d] Dissolved organic C and N
[e] Chloroform-extractable C and N
[f] Particulate organic matter

For inorganic N and N mineralization potential (N min), we sieved a subsample of soil in the field for inorganic N and 7-day anaerobic N mineralization incubation using a 2 *M* KCl extraction (Drinkwater et al. 1996). We analyzed Total NH_4^+ and NO_3^- using a continuous flow analyzer (AlpKem, OI Analytical, College Station, TX). We stored the remaining sieved and unsieved soils at 4° C. We completed dissolved organic C and N and microbial biomass extractions for each plot within 1 to 3 wk of field sampling on field moist samples using a modified chloroform fumigation-extraction method (Horwath and Paul 1994). Soil samples (15 g) were shaken for 4 h at 150 rpm in 40 mL 0.5 *M* K_2SO_4 with or without 0.5 mL chloroform, centrifuged and filtered through a 0.45 μm filter. Samples were lyophilized and analyzed for total C and N using a Vario El III CHNOS Elemental Analyzer (Elementar, Hanau, Germany). We present data as chloroform-extractable C and N since we did not use a K_c correction factor.

We separated fPOM and oPOM using size and density separation as in Marriott and Wander (2006). Briefly, fPOM was separated by floating on sodium polytungstate (1.7 g/ cm^3). The remaining soil sample was shaken with 10% sodium hexametaphosphate to disperse soil aggregates and then rinsed through a 53μm filter. The fraction larger than 53μm, which included sand and particulate organic matter, was retained. Total C and N of POM fractions and total soil were measured using a LECO 2000 CN Analyzer (Leco Corporation, St. Joseph, MO). To correct for carbonate content of each soil, a subset of samples from each field was treated with 6 *M* HCl and then analyzed for total C (Midwood and Boutton 1998). Carbonate C, the difference between untreated and HCl treated soil C, was subtracted from total soil C to calculate total soil organic C content for each field.

We sieved replicate composite samples of air-dried soil to 2mm for each field and analyzed for Morgan-extractable P, K, Ca, Mg, Cu, Zn, Fe, Al, and Mn (Cornell Nutrient Analysis Laboratory, Ithaca, NY) and particle size (Agricultural Analytical Services Laboratory, Penn State University, University Park, PA).

Statistical analysis

We computed statistics using JMP v.7 and SAS v.9.1 software (SAS Institute Inc., Cary, NC). Grain and no-grain treatments were compared using least squares means from mixed models including field and block as nested random factors. Least squares means from mixed models including field as a random factor were used to compare grain type and management type across fields. Multiple comparisons were calculated using Tukey's HSD and pairwise comparisons were calculated using

Student's *t*-tests. Correlations presented represent Pearson correlations of field means.

Due to the multicollinearity of soil variables, we used principal components analysis (PCA) to generate independent variables that represented soil texture and nutrient availability. Prior to conducting PCA, variables were assessed for normal distribution. Microbial biomass N, oPOM N, P and K were log-transformed to fit a normal distribution. Principal components analysis was conducted using a correlation matrix due to the different units and ranges of variables. A subset of C and N pools was selected to span the range from labile to more recalcitrant organic matter pools and C:N ratios were included to reflect organic matter quality. A subset of non-nitrogen soil nutrient variables and soil texture (sand, clay) was included that resulted in the greatest explained variability with the least redundancy. Principal components with eigenvalues greater than 1 and accounting for more than 10% of the variability in the data were retained. An orthogonal, Varimax rotation was used to improve the interpretability of the principal components. Principal component scores were then used as independent variables in mixed models to determine whether soil fertility explained the variability in plant response variables.

Results

Weather

Average temperatures during the 12 months of the experiment were similar to the 30-year average, with the exception of a warmer fall in 2006 (Figure 2.1a). Total recorded precipitation was 1000 mm for the study period, which was slightly less than normal. The precipitation distribution through the year differed from historical averages with drier periods at the beginning and end of the growing season and more precipitation than normal during the middle of the growing season. (Figure 2.1b).

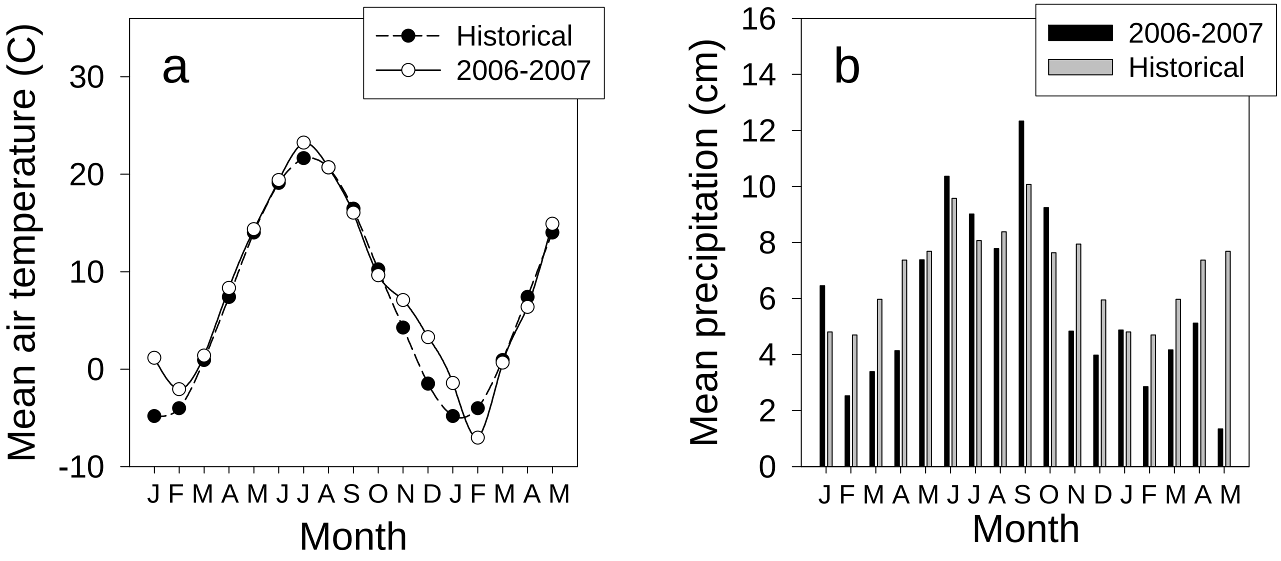


Figure 2.1. Monthly a) temperature average and b) precipitation sums for 2006-2007 study period and 30-year historical averages for Penn Yan and Aurora, New York.

Management history effects on soils

The 15 fields represented a fertility gradient due to management history and soil type. Both the quantity and quality of organic matter varied across the fields. Total organic carbon varied more than 2-fold across the fields from 13.5 to 29.3 mg g^{-1} (Table 2.4). Occluded POM C:N ranged from 15 to 24. Several soil variables differed by management history when soil clay concentration was included as a covariate. Phosphorus was higher in HBN than LEG fields and K followed a similar trend (Table 2.4). Inorganic N, fPOM N and oPOM N pools were larger in LEG fields. Differences in the quality of recent litter inputs are reflected in the greater N enrichment of fPOM in LEG fields (Table 2.4).

Principal components analysis resulted in three independent variables representing soil texture, N availability, and P and K availability (Figure 2.2). The three components explained 75% of the variability in the soils dataset. Textural variables (sand and clay) had the strongest loadings on principal component (PC) 1, which explained 31% of the variability (Table 2.5). Total organic carbon and Ca were highly correlated with clay concentration and also had high loadings on PC1. Soil carbon, chloroform-extractable N (MBN), and oPOM N were correlated with both PC1 and PC2, but had stronger loadings on PC2 (Figure 2.3). Labile organic matter quantity and quality and N availability were represented on PC2 with positive loadings for MBN, oPOM N, and inorganic N, and negative loadings for the C:N of oPOM and fPOM. The third PC explained 17% of the variability and had strong loadings for extractable P and K (Table 2.5).

Principal components analysis confirmed the influence of management history on soil N, P and K availability. Management history was not correlated with soil texture and the management types did not separate along PC1 (Figure 2.2). The fields separated by management type along PC2 and PC3. LEG fields had higher average

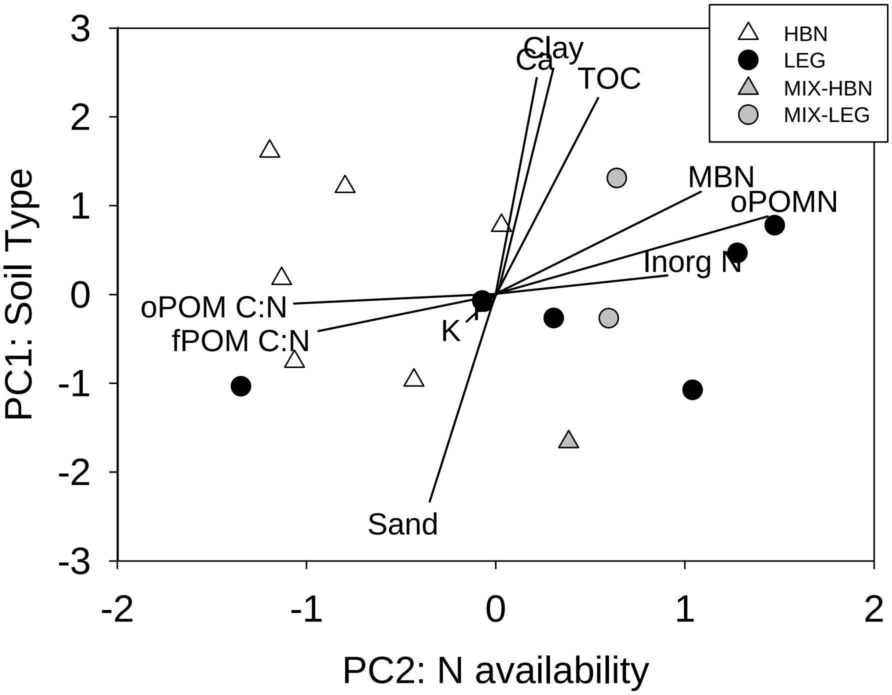


Figure 2.2. Bi-plot of principal component (PC) scores for each field and variable loadings (correlations between soil variables and PCs) for PC 1 and PC 2, which explained 31% and 27%, respectively, of total variability in the dataset. Haber-Bosch N-based systems (HBN) and legume N-based systems (LEG) are significantly different for PC 2 (p=0.03), but not for PC 1 (p>0.05). MIX-HBN = mixed HBN and legume N-based management history; MIX-LEG = mixed legume N and manure N-based management history

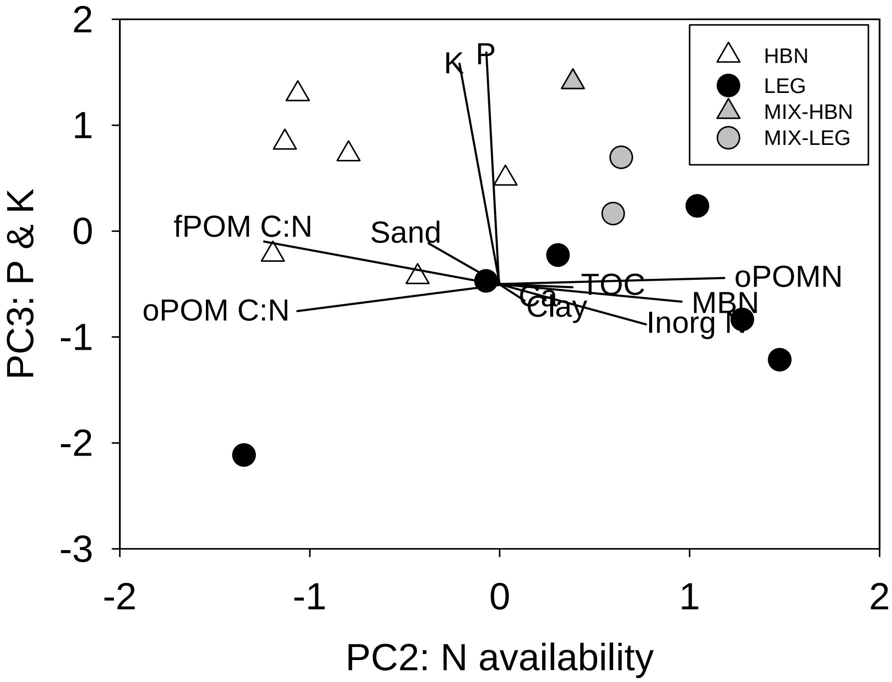


Figure 2.3. Bi-plot of principal component (PC) scores for each field and variable loadings (correlations between soil variables and PCs) for PC 2 and PC 3, which explained 27% and 17%, respectively, of the variability in the dataset. Haber-Bosch N-based systems (HBN) and legume N-based systems (LEG) are significantly different for both PC 2 and PC 3 (p=0.03 and p=0.01, respectively). MIX-HBN = mixed HBN and legume N-based management history; MIX-LEG = mixed legume N and manure N-based management history

Table 2.4. Subset of soil variables for each field and differences by management type. Fields are sorted from low to high N fertility as defined by principal components analysis. H=history of Haber-Bosch fertilizer N-based management; L=history of legume-based management; M=mixed HBN-legume or legume-manure management.

Field	TOC[a]	Sand	Clay	P	K	Ca	Inorg N[b]	MBN[c]	fPOM N[d]	oPOM N[e]	fPOM C:N	oPOM C:N
	——— g kg^{-1} ———			—— mg kg^{-1} ——			——— mg kg soil^{-1} ———					
L1	14.4	491	202	1.28	38	1218	2.41	4.80	23	42	26.7	23.7
H1	19.2	478	227	8.83	88	2531	2.63	6.39	16	38	25.8	23.1
H2	25.2	375	364	3.05	66	2645	3.87	8.32	25	58	27.3	22.9
H3	15.7	519	219	8.43	123	998	3.29	6.24	21	46	29.3	23.7
H4	22.8	373	351	5.38	90	2191	2.78	7.81	22	66	26.5	20.9
H5	13.5	488	226	3.83	66	1287	3.13	8.54	15	42	25.3	20.7
L2	17.8	472	276	2.50	77	2041	5.22	13.81	17	54	23.3	23.6
H6	20.3	371	325	6.70	71	2406	2.15	12.13	23	70	23.2	18.9
L3	17.2	421	264	3.03	76	1892	3.40	8.93	27	60	21.8	18.6
M1	14.0	543	169	9.25	124	645	3.04	9.58	27	56	25.2	19.5
M2	21.1	483	246	3.83	66	2281	3.14	6.91	26	88	24.6	15.1
M3	24.4	360	370	6.83	81	2623	4.31	14.78	32	78	20.9	18.4
L4	17.3	483	216	4.90	72	1415	5.09	6.86	27	90	24.1	16.8
L5	18.8	337	362	2.90	46	2023	4.29	15.00	30	93	21.2	14.7
L6	29.3	374	309	1.35	51	2322	5.93	22.41	30	116	21.0	18.5
SE[f]	1.0			0.57	4	135	0.48	0.88	3	6	0.8	0.7
Management effects[g]	ns	ns	ns	H>L	H>L	ns	L>H	ns	L>H	L>H	H>L	ns
p-value				0.02	0.07		0.04		0.03	0.04	0.01	

[a] Total organic carbon; [b] Extractable NO_3^- and NH_4^+ measured in June 2006; [c] Microbial biomass nitrogen;
[d] Free particulate organic matter nitrogen; [e] Occluded particulate organic matter nitrogen
[f] Pooled standard errors; [g] Student's *t*-test comparing H and L management categories, including soil clay as a covariate.

scores than HBN fields for PC2, indicating higher soil N availability (p=0.03; Figure 2.2). The LEG field with the lowest score on PC2 is the one transitional field included in the study which has been under organic management for only three years. HBN fields had higher average scores than LEG fields for PC3, indicating higher P and K availability (p=0.01; Figure 2.3).

Table 2.5. Rotated principal component eigenvalues, variation explained, and loadings for three principal components that explain a total of 75% of the variation in the soils dataset.

	PC1	**PC2**	**PC3**
Eigenvalue	3.4	2.9	1.9
Variation explained	31%	27%	17%
Rotated variable loadings			
Sand	-0.86	-0.27	0.21
Clay	0.92	0.17	-0.09
Ca	0.88	0.11	-0.05
Total organic C	0.81	0.33	0.00
log(MBN)[a]	0.44	0.65	-0.08
log(oPOM N)[b]	0.35	0.84	0.03
Inorg N[c]	0.12	0.57	-0.16
oPOM C:N	-0.05	-0.82	-0.11
fPOM[d] C:N	-0.18	-0.74	0.24
log(P)	-0.06	-0.04	0.96
log(K)	-0.14	-0.13	0.92

[a] Microbial biomass nitrogen; [b] Occluded particulate organic matter nitrogen;
[c] Extractable NO_3^- and NH_4^+ measured in June 2006; [d] Free particulate organic matter nitrogen

Species interactions and clover N fixation

Interseeded clover (IRC) average % N from fixation was similar to the % N from fixation of intercropped clover/ orchardgrass (RC/OG) and greater than monoculture clover (RC) and these differences remained in the spring (Figure 2.4). Interseeded clover had greater fall clover biomass production and N fixed than RC, despite a lower seeding rate (Figures 2.5a and 2.5b). Spring biomass production and N

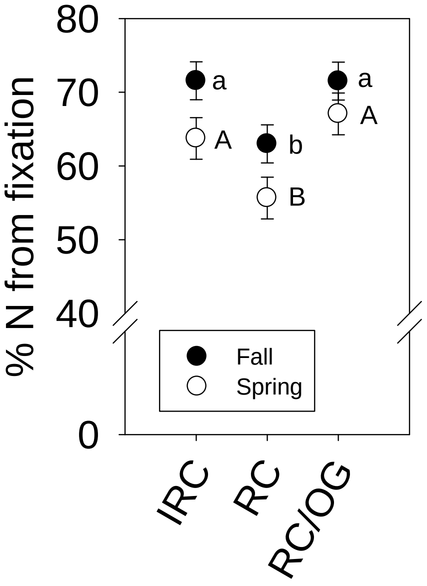


Figure 2.4. Fall and spring average % N from fixation for interseeded red clover (IRC), monoculture red clover (RC) and red clover grown in mixture with orchardgrass (RC/OG). Different letters across a sampling period indicate significant differences between treatments (p<0.05).

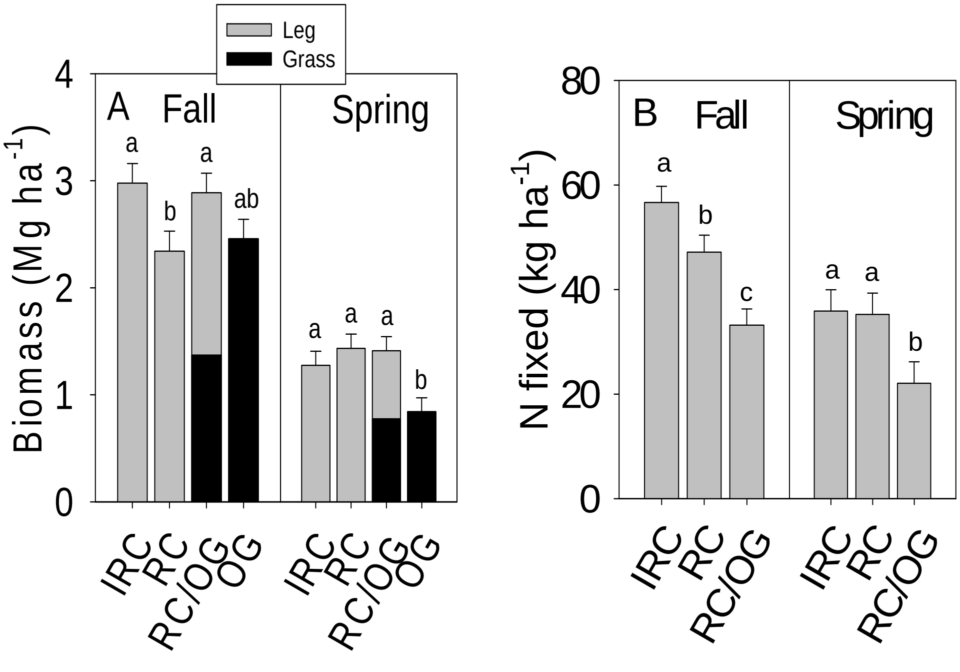


Figure 2.5. Fall and spring average (a) biomass and (b) N fixed for interseeded red clover (IRC), monoculture red clover (RC), red clover-orchardgrass mixtures (RC/OG), and orchardgrass monocultures (OG). Different letters across a sampling period indicate significant differences among treatments (p<0.05).

fixed did not differ between IRC and RC. Fall biomass N concentration of IRC was similar to RC/OG (Figure 2.6).

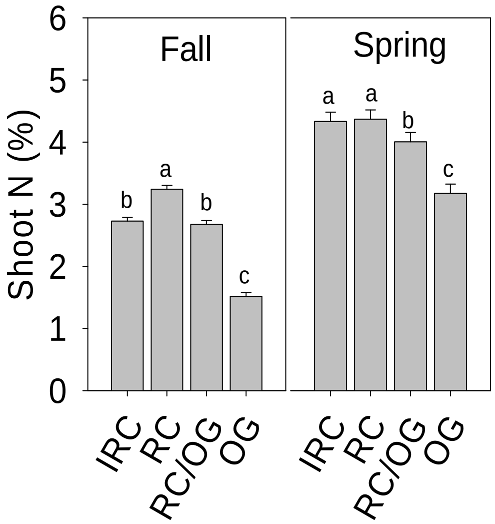


Figure 2.6. Fall and spring average aboveground biomass N concentration for interseeded red clover (IRC), monoculture red clover (RC), red clover grown in mixture with orchardgrass (RC/OG), and orchardgrass monoculture (OG). Different letters across a sampling period indicate significant differences among treatments ($p<0.05$).

Grain and clover interactions

The interseeded clover neither benefitted nor constrained grain production. Grain and straw N removal varied more than 2-fold across the study fields (Figure 2.7). Grain yields, grain N concentration, and total grain N did not differ between paired plots with and without clover (Table 2.6).

Grain type impacted fall clover biomass and N fixation. Management history and grain type interact because all spelt fields were fields under legume-based management. To control for management history effects on soil nutrient pools, the three PCs were included as covariates when comparing the effects of grain type on clover growth and N fixation. Despite large differences in grain biomass and grain N concentration between wheat and spelt, total N exported in grains did not differ by

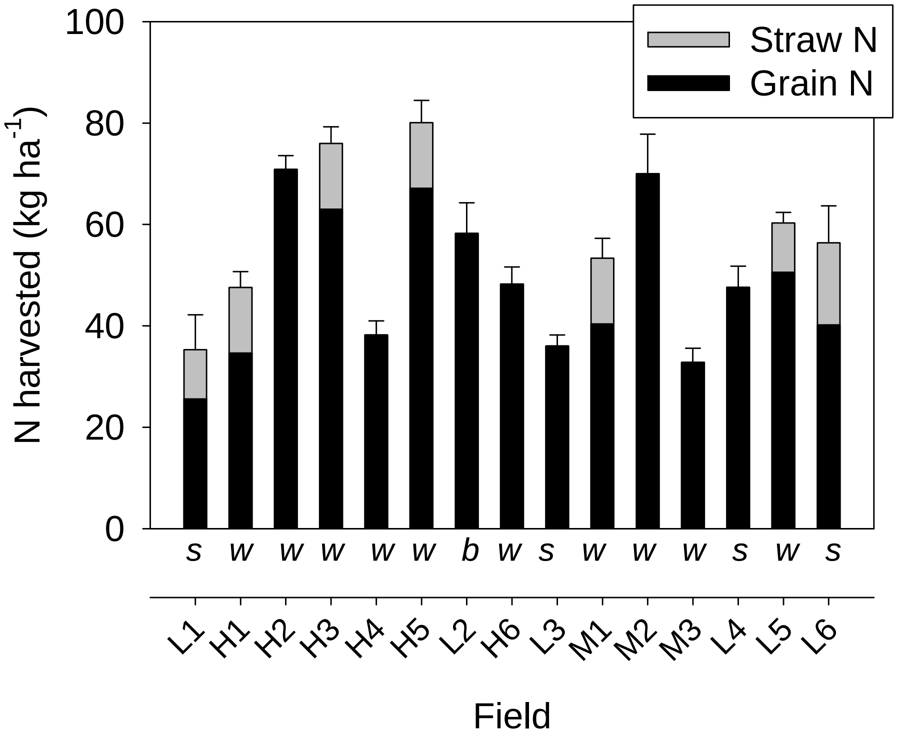


Figure 2.7. Nitrogen exported in grain and straw from spelt (s), winter wheat (w), and barley (b) crops. Error bars represent standard errors (n=8). Fields are sorted from low to high N fertility as defined by principal components analysis. H=history of Haber-Bosch fertilizer N-based management; L=history of legume-based management; M=mixed HBN-legume or legume-manure management.

Table 2.6. Least squares means for grain yield, N concentration, and total grain N in paired plots with and without clover.

	Yield	N	Grain N
Treatment	kg ha^{-1}	g kg^{-1}	kg ha^{-1}
Clover	2959	17.6	51
No clover	2906	17.6	49
Significance	ns	ns	ns

grain type (Table 2.7). Crop height was the most striking difference between spelt and wheat crops (Table 2.7). Controlling for differences in soil variables, fall clover biomass and N fixed were lower in spelt fields (Table 2.7). There was no residual effect of grain type on spring biomass and N fixation. There was also no effect of grain type on the % N from fixation in fall or spring.

Because the majority of LEG fields were planted to spelt and spelt fields had lower fall clover biomass and N fixation, the effects of management history on soil fertility and the effects of grain type on clover biomass coincided. Therefore, grain height was included as a covariate in models to study the effects of soil nutrient availability on fall clover variables. Barley was similar in height to wheat (70 cm).

Table 2.7. Least squares means for grain yield, grain N concentration, total N exported in grain, grain crop height, and average fall clover biomass and N fixed for spelt (n=4) and wheat (n=10) fields.

	Grain yield	Grain N	Grain N export	Grain crop height	Fall clover biomass	Fall clover N fixed
Crop	Mg ha^{-1}	g kg^{-1}	kg ha^{-1}	cm	Mg ha^{-1}	kg ha^{-1}
Spelt	1.91	20.5	39	128	2.04	35
Wheat	3.01	17.2	50	69	3.28	65
Significance	0.07	0.007	0.3	<.0001	0.02	0.02

Clover N fixation variability

Clover biomass and N fixation varied across the fields and the % of N from fixation was higher in fall than the following spring. Clover seeding date, which ranged from February 22 to April 21, did not affect fall clover biomass production ($p>0.05$). Fall biomass varied by over 300% from 1.3 to 4.2 Mg ha^{-1}. The N fixed was correlated with biomass in both the fall and the spring ($r=0.73$, $p=0.004$ and $r=0.89$, $p<.0001$, respectively). The relative reliance of clover on N fixation (% N from fixation) in the fall ranged from 53% to 88% and averaged 73%. Spring % N from fixation ranged from 35% to 78% and averaged 65% (Figure 2.8). Total N fixed in aboveground biomass averaged 57 kg N ha^{-1} in the fall and 36 kg N ha^{-1} in the spring. Spring clover biomass and N fixation measurements were not correlated with corresponding fall measurements. In contrast, fall and spring orchardgrass biomass N were strongly correlated ($r=0.78$, $p=0.001$).

The N fertility gradient did not explain the variability in clover N fixation. Positive correlations between both fall and spring orchardgrass N and PC2 provide evidence of a plant response to the N fertility gradient across field sites (Figure 2.9). In contrast, clover soil N uptake and N fixed were not correlated with soil N availability in fall or spring (Figure 2.9). Soil N availability also did not explain the variability in the % N from fixation in fall or spring.

Soil texture and the availability of P and K accounted for a significant proportion of the variability in fall clover soil N uptake and spring N fixation. Including grain crop height as a covariate, fall clover soil N uptake had a significant, negative relationship with PC1, indicating increased soil N uptake in sandier soils ($F=6.51$, $p=0.02$). PC1 was also a significant predictor of the % N from fixation in the spring ($F=6.86$, $p=0.01$), and the % N from fixation was higher in sandier soils. The variability in fall soil N uptake was also explained by PC3, suggesting P and K, in

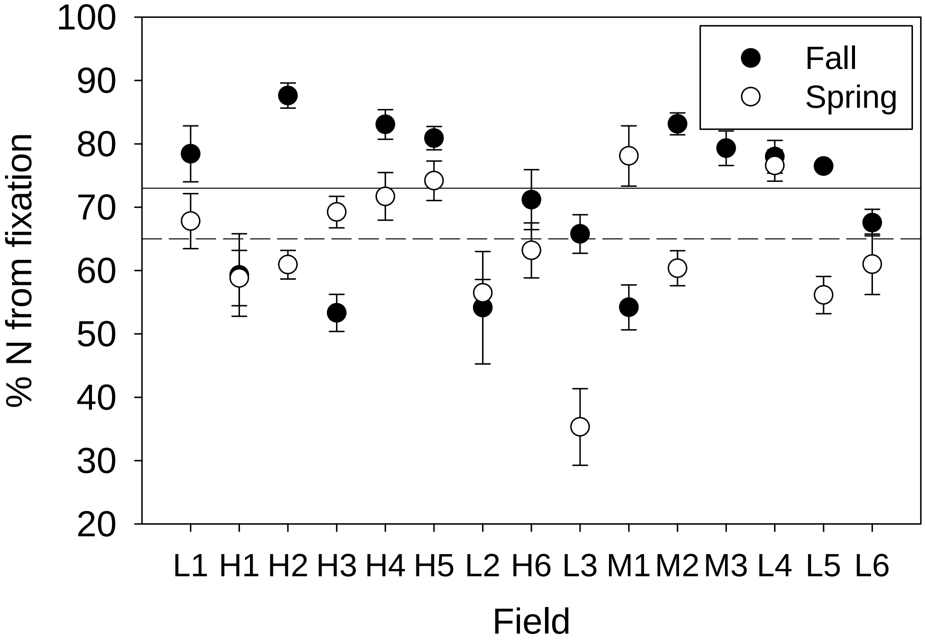


Figure 2.8. Clover interseeded with grain relative reliance on N fixation estimated in fall and spring clover aboveground biomass across 15 fields. Horizontal lines represent averages across all fields for fall (solid line) and spring (dashed line). Error bars represent standard errors (n=4). Fields are sorted from low to high N fertility as defined by principal components analysis. H=history of Haber-Bosch fertilizer N-based management; L=history of legume-based management; M=mixed HBN-legume or legume-manure management.

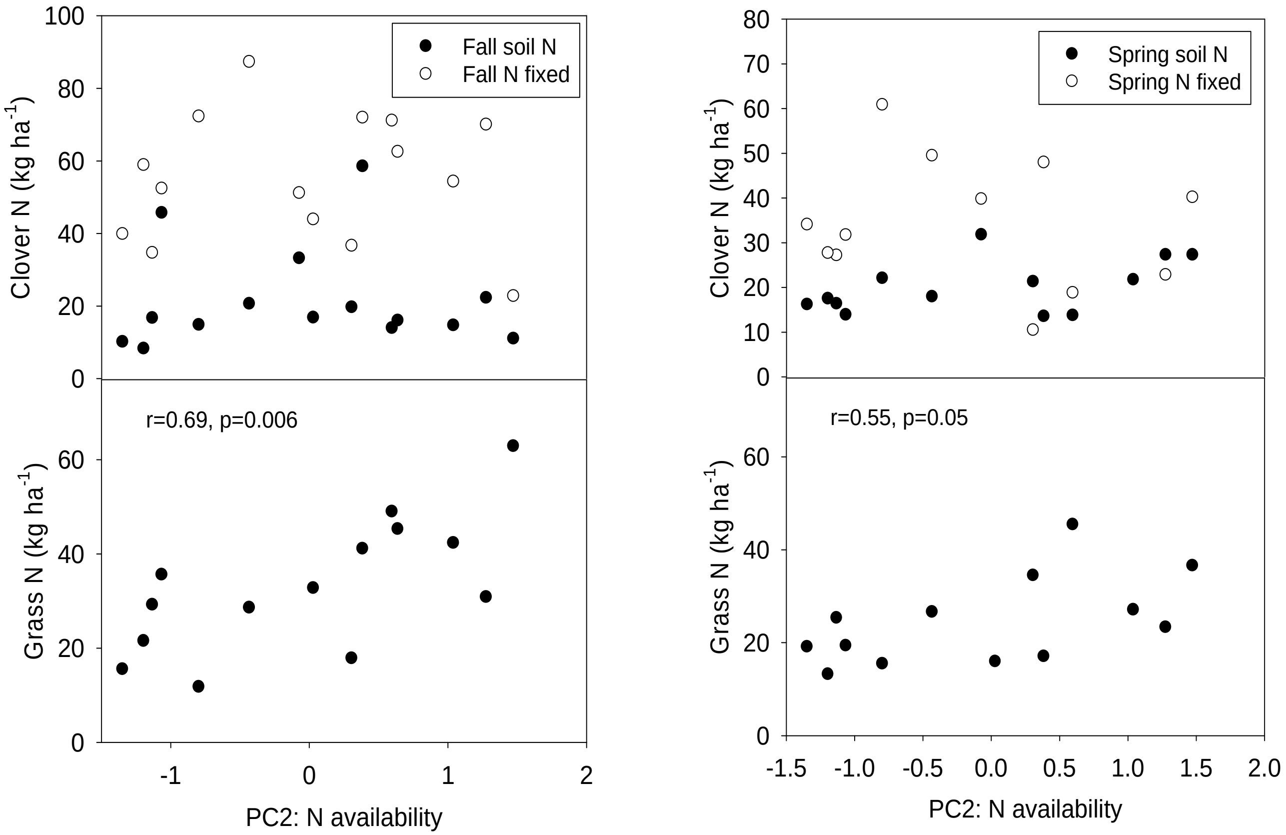


Figure 2.9. Correlations between fall and spring orchardgrass and clover biomass N and PC 2, reflecting soil N availability. Correlations are significant for spring and fall orchardgrass N assimilation, but not for spring or fall clover N fixed or soil N assimilation.

addition to soil texture, may have limited clover growth (F=7.40, p=0.01).

Discussion

We identified a management-induced N fertility gradient across farm fields. Fields under legume-based management had greater overall N availability as determined by principal components analysis (Figure 2.2). The differences in N availability were due to differences in both the quantity and quality of SOM pools. The differences in quality are reflected in the significant differences in fPOM C:N by management type (Table 2.4) and the strong loadings of fPOM C:N and oPOM C:N on PC2 (Table 2.5). These results support the finding of previous studies that have found a greater proportional retention of N inputs in legume-based systems than HBN-based systems (Clark et al. 1998; Drinkwater et al. 1998; Ross et al. 2008).

Soils data from the three fields that did not fall into our categories of legume-based or HBN-based management histories highlight important distinctions between legume-based, HBN-based, and manure-based systems due to the quantity and quality of N inputs. The coupling of C and N inputs in legume-based systems increases N retention and incorporation into stable soil aggregates, reducing its susceptibility to leaching losses in contrast to HBN fertilizer additions (Puget and Drinkwater 2001; Marriott and Wander 2006). The grouping of the one field with a history of HBN use and a high reliance on BNF for N inputs with the LEG fields along PC2 provides additional support for this conclusion (Figure 2.3). Manure-based systems also have C-N coupled inputs, but the quantity and fate of these inputs can be distinctly different than legume inputs. Manure-based systems tend to apply N, P, and K at levels that exceed exports (Nielsen and Kristensen 2005). The organically managed fields that relied on manure inputs for the majority of N inputs clustered with the HBN fields in terms of P and K availability, and with LEG fields in terms of N availability (Figure

2.3). Maintaining P and K fertility is a key challenge of legume-based systems (Berry et al. 2003).

Comparing relay crops, intercrops and monocultures

The relay cropping of clover interseeded into a grain combined the benefits of high reliance on N fixation of a mixture with the high legume biomass of a monoculture. Red clover interseeded with a winter grain fixed more N than both red clover monocultures and clover grown in mixture with a grass. Interseeded red clover had similar reliance on N fixation as the clover-orchardgrass mixture, which was higher than the clover monoculture (Figure 2.4). This suggests that the grain decreased soil N availability, increasing clover reliance on N fixation. The period of monoculture clover growth following grain harvest resulted in similar biomass accumulation in the interseeded and monoculture clovers (Figure 2.5), which is particularly interesting because the monoculture seeding rate was approximately 3-times greater than the interseeded clover seeding rate. Blaser et al. (2007) found small or no effect of seeding rate on clover biomass when interseeded with cereal grains. We observed distinct differences in interseeded clover stature compared to monoculture clover. The competition with the grain for light availability appeared to result in greater investment in vertical growth for interseeded clover. This is reflected in the lower N concentration of interseeded clover relative to monocultures (Figure 2.6).

Top-down grain effects on clover growth

Winter grain type had strong top-down effects on clover biomass. Spelt fields had lower clover biomass and total N fixed at the end of the first year of growth (Table 2.7). Reduced clover biomass in spelt fields was likely due to light competition from taller spelt plants (Klebesadel and Smith 1959). Because the spelt fields were all legume-based fields that had higher N availability and lower P and K availability on average, it is difficult to separate the effects of soil fertility and grain type on clover dynamics. We did not find any evidence of P and K limitation in monoculture clover growth, but total biomass accumulation in RC/OG mixtures was positively correlated with P and K availability (Chapter 3).

Farmers have observed the influence of crop stature on clover biomass. In years with higher than normal precipitation prior to grain harvest, red clover biomass accumulation can interfere with harvest of shorter stature wheat varieties. Some farmers prefer using taller varieties of wheat or spelt for intercropping with clover because their taller stature reduces the risk of clover interference with grain growth and harvest in wet years (Fred Sepe and Klaas Martens, personal communications). Clover competition with grain harvest was not a problem during the year when this study was conducted.

Soil fertility effects on clover N fixation

We found no evidence of soil N availability inhibiting N fixation across the fertility gradient. Nitrogen additions typically exceed N harvests from farm fields in this region, placing these fields on the upper end of a scale of N availability ranging from N limited to N saturated (Drinkwater and Snapp 2007). The presence of a N fertility gradient across sites was confirmed by the positive correlation between orchardgrass N accumulation and PC2 (Figure 2.9). The lack of a response of N fixation to the N fertility gradient was not isolated to the IRC treatment. Monoculture RC N fixation also was not correlated with N fertility (Chapter 3). The RC/OG mixture showed a small response, with a weak correlation between PC2 and red clover biomass relative to total mixture biomass (Chapter 3). In a separate study, we found a slightly stronger influence of soil N availability across a similar set of sites and soybean (*Glycine max*) N fixation, but soil texture was a much stronger driver of soybean N fixation than N availability (Chapter 1).

The accepted concept that soil N availability is a dominant edaphic factor influencing legume N fixation has rarely been tested across a gradient of N availability driven by the mineralization of endogenous SOM pools. Most research has focused on the response of N fixation to recent, short-term pulses of labile N additions through the addition of HBN fertilizers at different rates (Boller and Nosberger 1994; Hogh-Jensen and Schjoerring 1997). The only study we are aware of that measured N fixation response to soil N availability on farms without recent fertilizer additions found a negative relationship between soil nitrate and legume N fixation (Schwenke et al. 1998). However, the soil nitrate levels measured by Schwenke et al. (1998) on farms in Australia following fallow periods were more than an order of magnitude larger than the levels we measured, which are within a typical range for farms in the Northeast. Our results suggest that N fixation is not sensitive to the range of soil N availability driven by mineralization of organic matter in these systems.

While grass biomass accumulation was primarily N limited, clover biomass accumulation was affected by soil texture and P and K availability. In agreement with other studies, lighter textured soils supported improved legume growth and N fixation (Chapter 1 (Riffkin et al. 1999). Textural effects on BNF may be a result of increased frequency of water saturated conditions in finer textured soils, which can inhibit BNF and root and soil biological activity. Soil textural effects on soil moisture and aeration can also affect plant pathogens. Finer-textured soils may have increased prevalence of legume root pathogens (Workneh et al. 1999).

Estimating N fixation inputs

Estimating clover N fixation inputs requires improved tools and additional research on relationships between clover physiology and N fixation. Clover N fixation was positively correlated with total biomass for both fall and spring measurements. Developing tools for farmers to quickly estimate clover biomass could lead to improved N management through more accurate accounting of N inputs. For example, if the average measurement of 73% N from fixation was used in conjunction with accurate aboveground biomass estimates across sites, N fixation estimates would be within ± 20 kg N ha^{-1} of our fall measurements for all fields.

Estimates of red clover root biomass are another area of uncertainty in N fixation inputs for calculating legume-based agroecosystem N balances. The development of ^{15}N shoot-labelling techniques to quantify belowground plant N has led to much higher estimates than standard root sampling methods. For example, Hogh-Jensen and Schjoerring (2001) measured approximately 60% of total red clover N in belowground roots and rhizodeposits within the first year. Root estimates need to be incorporated into estimates of legume N inputs when calculating N balances.

Due to the translocation and recycling of fall fixed N inputs into spring clover biomass, spring N fixation inputs remain poorly understood. Fall aboveground plant N is either translocated into plant roots, incorporated into soil organic matter, or lost from the system. The translocation of N from roots to shoots during regrowth has been estimated to account for half of annual aboveground N in perennial grasses (Blair et al. 1998). Losses of 30-40% for fall shoot N from white clover (*Trifolium repens*) over the winter have been measured, with approximately 40% of this N being captured in soil and available for possible uptake again during spring growth (Sturite et al. 2006; Sturite et al. 2007). The ^{15}N natural abundance method is unable to differentiate recent fixed N in spring growth from N fixed the previous fall. Spring measurements provide a useful estimate of labile N additions that may become rapidly available for the following crop. More research is needed to understand the overwinter dynamics of N cycling in perennial legumes.

Conclusion

Legume-based management increased soil N availability, but we found no evidence that increased N availability serves as an internal regulator of BNF in legume-based cropping systems. Interseeding red clover into winter grain increased the relative reliance of clover on N fixation due to the short period of competitive plant species interactions while retaining high biomass production due to the period of monoculture growth following grain harvest. Winter grain type had a strong top-down effect on clover growth and, consequently, on N fixation during the establishment year. Diversifying grain rotations to include winter grains provides an excellent niche for the inclusion of legume cover crops that can serve multiple ecosystem services.

REFERENCES

Berry PM, Stockdale EA, Sylvester-Bradley R, Philipps L, Smith KA, Lord EI, Watson CA, Fortune S (2003) N, P and K budgets for crop rotations on organic farms in the UK. Soil Use and Management 19:112-118

Blair JM, Seastedt TR, Rice CW, Ramundo RA (1998) Terrestrial nutrient cycling in tallgrass prairie. In: Knapp AK, Briggs JM (eds) Grassland Dynamics: Long-Term Ecological Research in Tallgrass Prairie. Oxford University Press, New York

Blaser BC, Gibson LR, Singer JW, Jannink JL (2006) Optimizing seeding rates for winter cereal grains and frost-seeded red clover intercrops. Agronomy Journal 98:1041-1049

Blaser BC, Singer JW, Gibson LR (2007) Winter cereal, seeding rate, and intercrop seeding rate effect on red clover yield and quality. Agronomy Journal 99:723-729

Bliss FA (1993) Breeding common bean for improved biological nitrogen-fixation. Plant and Soil 152:71-79

Boller BC, Nosberger J (1994) Differences in nitrogen-fixation among field-grown red clover strains at different levels of ^{15}N fertilization. Euphytica 78:167-174

Boone RD (1994) Light-fraction soil organic matter: origin and contribution to net nitrogen mineralization. Soil Biology & Biochemistry 26:1459-1468

Bruulsema TW, Christie BR (1987) Nitrogen contribution to succeeding corn from alfalfa and red clover. Agronomy Journal 79:96-100

Bula RJ, Smith D, Miller EE (1954) Measurements of light beneath a small-grain companion crop as related to legume establishment. Botanical Gazette 115:271

Carlsson G, Huss-Danell K (2003) Nitrogen fixation in perennial forage legumes in the field. Plant and Soil 253:353-372

Clark MS, Horwath WR, Shennan C, Scow KM (1998) Changes in soil chemical properties resulting from organic and low-input farming practices. Agronomy Journal 90:662-671

Drinkwater LE, Cambardella CA, Rice CW (1996) Potentially mineralizable N as an indicator of active soil N. In: Doran J, Jones AJ (eds) Methods for assessing soil quality. Soil Science Society of America, Madison, WI

Drinkwater LE, Snapp SS (2007) Nutrients in agroecosystems: rethinking the management paradigm. Advances in Agronomy 92:163-186

Drinkwater LE, Wagoner P, Sarrantonio M (1998) Legume-based cropping systems have reduced carbon and nitrogen losses. Nature 396:262-265

Elgersma A, Schlepers H, Nassiri M (2000) Interactions between perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) under contrasting nitrogen availability: productivity, seasonal patterns of species composition, N_2 fixation, N transfer and N recovery. Plant and Soil 221:281-299

Fageria NK, Santos AB (2008) Yield physiology of dry bean. Journal Of Plant Nutrition 31:983-1004

Gan YB, Stulen I, van Keulen H, Kuiper PJC (2002) Physiological changes in soybean (Glycine max) Wuyin9 in response to N and P nutrition. Annals of Applied Biology 140:319-329

Goss MJ, de Varennes A, Smith PS, Ferguson JA (2002) N_2 fixation by soybeans grown with different levels of mineral nitrogen, and the fertilizer replacement value for a following crop. Canadian Journal of Soil Science 82:139-145

Hardarson G, Bliss FA, Cigalesrivero MR, Henson RA, Kipenolt JA, Longeri L, Manrique A, Penacabriales JJ, Pereira PAA, Sanabria CA, Tsai SM (1993)

Genotypic variation in biological nitrogen-fixation by common bean. Plant and Soil 152:59-70

Hauggaard-Nielsen H, Jornsgaard B, Kinane J, Jensen ES (2008) Grain legume-cereal intercropping: The practical application of diversity, competition and facilitation in arable and organic cropping systems. Renewable Agriculture and Food Systems 23:3-12

Hogh-Jensen H, Loges R, Jorgensen FV, Vinther FP, Jensen ES (2004) An empirical model for quantification of symbiotic nitrogen fixation in grass-clover mixtures. Agricultural Systems 82:181-194

Hogh-Jensen H, Schjoerring JK (1997) Interactions between white clover and ryegrass under contrasting nitrogen availability: N-2 fixation, N fertilizer recovery, N transfer and water use efficiency. Plant and Soil 197:187-199

Hogh-Jensen H, Schjoerring JK (2001) Rhizodeposition of nitrogen by red clover, white clover and ryegrass leys. Soil Biology & Biochemistry 33:439-448

Horwath WR, Paul EA (1994) Microbial biomass. In: Weaver R, Angle JS, Bottomley PS (eds) Methods of soil analysis. Part 2---microbiological and biochemical properties. Soil Science Society of America, Madison, WI, pp 753-773

Klebesadel LJ, Smith D (1959) Light and soil moisture beneath several companion crops as related to the establishment of alfalfa and red clover. Botanical Gazette 121:39

Lecoeur J, Sinclair TR (2001) Harvest index increase during seed growth of field pea. European Journal of Agronomy 14:173-180

Lotter DW, Seidel R, Liebhardt W (2003) The performance of organic and conventional cropping systems in an extreme climate year. American Journal of Alternative Agriculture 18:146-154

Marriott EE, Wander MM (2006) Total and labile soil organic matter in organic and conventional farming systems. Soil Science Society of America Journal 70:950-959

Mayer J, Buegger F, Jensen ES, Schloter M, Hess J (2003) Estimating N rhizodeposition of grain legumes using a N-15 in situ stem labelling method. Soil Biology & Biochemistry 35:21-28

Midwood AJ, Boutton TW (1998) Soil carbonate decomposition by acid has little effect on delta ^{13}C of organic matter. Soil Biology & Biochemistry 30:1301-1307

Nielsen A, Kristensen I (2005) Nitrogen and phosphorun surpluses on Danish dairy and pig farms in relation to farm characteristics. Livestock Production Science 96:97-107

Puget P, Drinkwater LE (2001) Short-term dynamics of root- and shoot-derived carbon from a leguminous green manure. Soil Science Society of America Journal 65:771-779

Riffkin PA, Quigley PE, Kearney GA, Cameron FJ, Gault RR, Peoples MB, Thies JE (1999) Factors associated with biological nitrogen fixation in dairy pastures in south-western Victoria. Australian Journal of Agricultural Research 50:261-272

Rochester IJ, Peoples MB, Constable GA, Gault RR (1998) Faba beans and other legumes add nitrogen to irrigated cotton cropping systems. Australian Journal of Experimental Agriculture 38:253-260

Ross SM, Izaurralde RC, Janzen HH, Robertson JA, McGill WB (2008) The nitrogen balance of three long-term agroecosystems on a boreal soil in western Canada. Agriculture Ecosystems & Environment 127:241-250

Schwenke GD, Peoples MB, Turner GL, Herridge DF (1998) Does nitrogen fixation of commercial, dryland chickpea and faba bean crops in north-west New South Wales maintain or enhance soil nitrogen? Australian Journal of Experimental Agriculture 38:61-70

Shearer G, Kohl DH (1986) N_2 fixation in field settings: estimates based on natural ^{15}N abundance. Australian Journal of Plant Physiology 13:699-756

Snapp SS, Swinton SM, Labarta R, Mutch D, Black JR, Leep R, Nyiraneza J, O'Neil K (2005) Evaluating cover crops for benefits, costs and performance within cropping system niches. Agronomy Journal 97:322-332

Sturite I, Henriksen TM, Breland TA (2007) Winter losses of nitrogen and phosphorus from Italian ryegrass, meadow fescue and white clover in a northern temperate climate. Agriculture Ecosystems & Environment 120:280-290

Sturite I, Uleberg MV, Henriksen TM, Jorgensen M, Bakken AK, Breland TA (2006) Accumulation and loss of nitrogen in white clover (*Trifolium repens* L.) plant organs as affected by defoliation regime on two sites in norway. Plant and Soil 282:165-182

Tonitto C, David MB, Drinkwater LE (2006) Replacing bare fallows with cover crops in fertilizer-intensive cropping systems: A meta-analysis of crop yield and N dynamics. Agriculture Ecosystems & Environment 112:58-72

Vandermeer J (1989) The ecology of intercropping. Cambridge University Press, Cambridge

Vyn TJ, Janovicek KJ, Miller MH, Beauchamp EG (1999) Soil nitrate accumulation and corn response to preceding small-grain fertilization and cover crops. Agronomy Journal 91:17-24

Wander M (2004) Soil organic matter fractions and their relevance to soil function. In: Magdoff F, Weil RR (eds) Soil organic matter in sustainable agriculture. CRC Press, Boca Raton, FL

Watson CA, Bengtsson H, Ebbesvik M, Loes AK, Myrbeck A, Salomon E, Schroder J, Stockdale EA (2002) A review of farm-scale nutrient budgets for organic farms as a tool for management of soil fertility. Soil Use and Management 18:264-273

Workneh E, Yang XB, Tylka GL (1999) Soybean brown stem rot, *Phytophthora sojae*, and *Heterodera glycines* affected by soil texture and tillage relations. Phytopathology 89:844-850

Zhang FS, Li L (2003) Using competitive and facilitative interactions in intercropping systems enhances crop productivity and nutrient-use efficiency. Plant and Soil 248:305-312

CHAPTER THREE

LEGUME NITROGEN FIXATION ACROSS A FERTILITY GRADIENT: THE EFFECTS OF PLANT PHENOLOGY AND SPECIES INTERACTIONS

Abstract

The selection of legume cover crop species and species mixtures can influence agroecosystem nitrogen (N) and carbon cycling. We utilized a management-driven fertility gradient across 15 farm fields to study the ecology of N fixation of an annual legume (*Pisum sativum*) and perennial legume (*Trifolium pratense*) grown in monoculture and mixtures with orchardgrass (*Dactylis glomerata*) or oats (*Avena sativa*). Evidence of complementary and facilitative species interactions was stronger for the perennial red clover-orchardgrass mixture than for the annual field pea-oat mixture. The average Land Equivalency Ratios were 1.6 and 1.2 for perennial and annual mixtures, respectively. We estimated that the transfer of fixed N from red clover to orchardgrass increased total aboveground N fixation by an average of 5 kg N ha^{-1}, representing a 15% increase in N fixation estimates. Mixtures, however, did not exhibit increased stability of biomass production across field sites. Biomass yield of monoculture red clover was more stable across field sites than the other plant treatments. Despite a more than 2-fold range in soil organic matter levels and more than 6-fold range in soil N assimilation by grasses across field sites, the management-induced N fertility gradient was not a strong predictor of N fixation. While grass N assimilation was positively correlated with soil N availability, we found only weak correlations between legume N fixation and soil N availability. The longer growth period of perennials may have increased their ability to respond to environmental conditions through feedback mechanisms. These results suggest that increasing diversity of cropping systems, particularly the incorporation of perennials into

rotations, could improve overall agroecosystem N cycling efficiency and yield stability.

Introduction

The industrialization of agriculture relies on external fertilizer inputs to regulate nitrogen (N) availability, reducing agroecosystem reliance on internal nutrient cycling dynamics (Woodmansee 1984). The application of synthetic N fertilizers in plant available forms and the adoption of less diverse crop rotations have reduced plant cover in time and space and uncoupled carbon (C) and N cycles. As a result of this uncoupling of C and N cycles, synthetic N additions are needed at levels that often saturate the system (Drinkwater and Snapp 2007). Large synthetic N additions combined with the absence of living plant cover between harvested crops have resulted in large leaching losses of N to surface waters (Galloway and Cowling 2002; McIsaac et al. 2001; Mitsch et al. 2001)

Cropping systems that rely on C-N coupled inputs from legume biological nitrogen fixation (BNF) rely on complex plant-soil-microbe interactions to regulate N cycling. Legume cover crops increase the time of soil coverage and net primary productivity (NPP), resulting in increased C fixation, reduced soil erosion, and weed suppression, in addition to improving N availability and retention (Ranells and Wagger 1997; Tonitto et al. 2006). Despite the multiple functions that legume cover crops support, N management research has focused primarily on adjusting applications of synthetic N fertilizer to improve N retention (Gardner and Drinkwater, in press). Improving our understanding of the ecology of legume cover crops is critical to designing sustainable agroecosystems that rely on plant and microbially-mediated N cycling from SOM reservoirs and BNF inputs.

In natural systems, soil N availability is one of the primary factors thought to constrain the distribution of legumes due to the suppressive effect of soil N availability on BNF (Vitousek et al. 2002). However, the relationship between endogenous soil N reservoirs and BNF has been studied only rarely because inorganic N additions typically are used to produce N-fertility gradients (Elgersma et al. 2000; Nesheim and Oyen 1994). In systems where N availability is driven by soil organic matter dynamics, plant species interact with the soil environment through complex plant-soil-microbe interactions.

The effects of competitive and facilitative interactions on BNF in grass-legume mixtures vary at different levels of soil N availability. Including legume-grass mixtures in agricultural systems may increase the yield stability and N cycling efficiency of cover crops in response to shifting resource availability (Malezieux et al. 2009). The ability of grasses to effectively scavenge soil N can improve total N retention and recycling in legume-grass mixtures relative to monocultures while still maintaining modest additions of fixed N. Competitive interactions in mixtures can increase legume reliance on N fixation, compared with legume monocultures, even under conditions with relatively high soil N availability because grasses out-compete legumes for available soil N (Hogh-Jensen and Schjoerring 1997; Munoz and Weaver 1999). However, competitive interactions can result in a net decrease in total N fixed despite greater N fixation rates when grasses suppress legume biomass production (Boller and Nosberger 1994; Elgersma et al. 2000). Finally, the transfer of fixed N from legume to grass can facilitate grass NPP in legume-grass mixtures when N is limiting (Hogh-Jensen and Schjoerring 2000). The benefits of mixtures for total N retention and N fixation additions may depend on background soil N availability.

Annual and perennial legume BNF may respond differently to a gradient of soil N availability. Annual and perennial legume responses to soil N availability have not been directly compared within a single study. However, cross-study comparisons suggest that N fixation of annual grain soybeans is more sensitive to N fertilization than it is for perennial legumes such as red clover and alfalfa (Boller and Nosberger 1994; Hardarson et al. 1984; Lamb et al. 1995). Intensive breeding selection of annual grain legumes under high soil N conditions may have affected their ability to effectively down-regulate BNF as soil N availability increases compared with perennial forage legumes (Kiers et al. 2007).

We utilized a management-driven fertility gradient across 15 farm fields to study the ecology of N fixation of an annual and perennial legume grown in monoculture and mixtures with a grass. Our objective was to investigate the effects of plant phenology and plant species interactions on net primary productivity (NPP) and BNF responses to shifting soil resource availability.

Materials and Methods

Experimental sites

Research plots were established in 2006 in 15 fields on 7 commercial grain farms in central New York state (42° 36’-42° 44’ N and 77° 03’-76° 42’ W). Fields were selected to reflect a gradient of soil nitrogen availability due to differences in management history (Table 3.1). Full site descriptions and weather data can be found in Chapter 2.

Briefly, 5-year management histories were used to classify each field based on the percentage of total N inputs derived from either HBN fertilizers or legume BNF inputs. Nitrogen management categories were defined as: 1) >50% N inputs from HBN (HBN); and 2) >50% N inputs from legume BNF (LEG). All L fields were certified organic, ranging from 3 to 17 years since conversion to organic management

Table 3.1. Field management history, including years under organic management, % of N inputs from legume BNF over 5-year rotation cycle, crop rotation, and soil type.

Field[a]	**Years Org**	**% N inputs from BNF**	**5-year rotation**[b]	**Soil classification**
H1	0	8	corn-corn-corn-kidney beans-wheat/clover	Fine sandy-loam, Oxyaquic Hapludalf
H2	0	34	corn-soybean-corn-snap bean-wheat	Silt loam, Oxyaquic Hapludalf
H3	0	49	snap beans-wheat/clover-corn-snap beans-wheat/clover	Fine sandy-loam, Oxyaquic Hapludalf
H4	0	33	soybean-corn-corn-soybean-wheat	Silt loam, Oxyaquic Hapludalf
H5	0	49	snap beans-wheat/clover-corn-snap beans-wheat/clover	Fine sandy-loam, Oxyaquic Hapludalf
H6	0	16	hay-corn-corn-snap beans-wheat	Silt loam, Oxyaquic Hapludalf
M1	0	60	soybean-wheat/clover-corn-soybean-wheat/clover	Gravelly loam, Glossic Hapludalf
M2	16	46	soybean-wheat/clover-corn-kidney bean-spelt/clover	Fine silt loam, Glossic Hapludalf
M3	6	42	fallow-clover-corn-fallow-wheat/clover	Silt loam, Oxyaquic Hapludalf
L1	3	62	hay-hay-soybean-fallow-spelt/clover	Silt loam, Oxyaquic Hapludalf
L2	12	65	soybean-spelt/clover-corn-oats/peas-barley/clover	Fine silt loam, Glossic Hapludalf
L3	16	57	cabbage-spelt/clover-corn-kidney bean-wheat/clover	Fine silt loam, Glossic Hapludalf
L4	7	88	spelt/clover-cabbage-clover-clover-spelt/clover	Fine sandy-loam, Glossic Hapludalf
L5	17	60	soybean-wheat/clover-corn-soybeans-wheat/clover	Silt loam, Oxyaquic Hapludalf
L6	7	68	hay-soybean-wheat/clover-snap beans-spelt/clover	Gravelly silt loam, Glossaquic Hapludalf

[a] H=Haber-Bosch fertilizer N-based management; M=mixed HBN-legume or legume-manure management; L=legume-based management

[b] Cropping years are separated by dashes and intercroppings are separated by slashes with 2006 crop on right. Corn (*Zea mays*); kidney bean and snap bean (*Phaseolus vulgaris*); rye (*Secale cereale*); wheat (*Triticum aestivum*); spelt (*Triticum spelta*); clover (*Trifolium pratense*); oats (*Avena sativa*); peas (*Pisum sativum*); cabbage (*Brassica oleracea*)

practices (Table 3.1). Three fields did not fit into either management category because they either used HBN, but relied on BNF for more than 50% of N inputs (Field M1), or did not use HBN, but relied on BNF for less than 50% of N inputs with the remainder of N inputs from manure inputs (Fields M2 and M3). These three fields were lumped into a mixed category (MIX).

Soil sampling and analysis

Soil sampling and analysis details are described in Chapter 2. Here, we briefly summarize our approach and the findings used to analyze plant responses to soil characteristics.

We collected soil samples at three time points during 2006. Between June 7 and June 15, we sampled soils across each replicate block (Sampling 1). Eighteen soil cores (2-cm diameter by 20-cm depth) were taken and composited from each block within each field. Between August 9 and August 11, we sampled soils following winter grain harvest (Sampling 2). Six soil cores (2-cm diameter by 20-cm depth) were taken and composited from each block within each field. In October, we sampled soils from each individual subplot. We used 7-cm Dutch augers to collect approximately 2 kg of soil from each plot to 20-cm depth. To measure soil bulk density, we collected four soil cores of exactly 2-cm diameter by 20-cm depth from 8 randomly selected plots within each field.

A range of soil N fractions was quantified to span the continuum from labile to more recalcitrant N pools based on their availability for microbial mineralization. Chapter 2 provides a full description of soil fractions measured and methods used. Briefly, C and N pools measured included total soil C and N, free and occluded particulate organic matter (fPOM and oPOM), microbial biomass N (MBN), N mineralization potential, and inorganic N. In addition, we analyzed soils for Morgan-extractable P, K, Ca, Mg, Cu, Zn, Fe, Al, and Mn (Cornell Nutrient Analysis Laboratory, Ithaca, NY) and particle size (Agricultural Analytical Services Laboratory, Penn State University, University Park, PA).

Due to the multicollinearity of soil variables, we used principal components analysis (PCA) to generate independent variables that represented soil texture and nutrient availability. As described in Chapter 2, PCA resulted in three independent variables representing soil texture (PC1), N availability (PC2), and P and K availability (PC3) (Table 3.2). The fields separated by management type along PC2 and PC3. LEG fields had higher average scores than HBN fields for PC2, indicating higher soil N availability (Chapter 2). HBN fields had higher average scores than LEG fields for PC3, indicating higher P and K availability.

Table 3.2. Rotated principal component eigenvalues, variation explained, and loadings for three principal components that explain a total of 75% of the variation in the soils dataset.

	PC1	**PC2**	**PC3**
Eigenvalue	3.4	2.9	1.9
Variation explained	31%	27%	17%
Rotated variable loadings			
Sand	-0.86	-0.27	0.21
Clay	0.92	0.17	-0.09
Ca	0.88	0.11	-0.05
Total organic C	0.81	0.33	0.00
log(MBN)	0.44	0.65	-0.08
log(oPOM N)	0.35	0.84	0.03
Inorg N	0.12	0.57	-0.16
oPOM C:N	-0.05	-0.82	-0.11
fPOM C:N	-0.18	-0.74	0.24
log(P)	-0.06	-0.04	0.96
log(K)	-0.14	-0.13	0.92

[a] Microbial biomass nitrogen; [b] Occluded particulate organic matter nitrogen;
[c] Extractable NO_3^- and NH_4^+ measured in June 2006; [d] Free particulate organic matter nitrogen

Plot establishment and management

Legume cover crop niches vary for temperate cropping systems. In annual grain systems, perennial species are integrated into rotations as either forages or as green manures to increase N availability for a subsequent crop. In annual vegetable systems, winter-killed annual cover crop species are more commonly integrated into rotations to provide soil cover through the winter without conflicting with early spring planting dates. We utilized realistic cover crop niches in annual grain agroecosystems to measure the BNF of red clover (*Trifolium pratense*), a perennial legume commonly integrated into annual grain systems, and field pea (*Pisum sativum*), an annual legume cover crop used in annual vegetable systems, grown in monoculture and mixture with a grass.

We established six plant treatments in a split-plot design with four replicate blocks per field. All collaborating farmers used conventional tillage practices and had seeded all fields in the study to a winter grain in the fall of 2005. Plots measured 1.5-m by 3.0-m. For perennial plots, spring winter grain growth was removed using flame weeding in LEG fields and field M1, or glyphosate treatment in HBN fields and fields M2 and M3, in March and April 2006. We mowed and removed senescent aboveground biomass from herbicide-treated fields to produce similar starting conditions in all plots. Into cleared plots, we broadcast perennial ‘Medium’ red clover and orchardgrass (*Dactylis glomerata*) as monocultures and mixture during May 5-7, 2006, at the rates of 35 kg/ha for monoculture red clover (RC), 30 kg/ha for monoculture orchardgrass (OG), and 20 kg/ha for red clover and 15 kg/ha for orchardgrass in mixed plots (RC/OG). Annual plots were established adjacent to perennial plots following winter grain harvest. In July 2006, we rototilled remaining grain stubble and drill-seeded annual ‘Maxim’ field peas and oats (*Avena sativa*) as monocultures and mixture on August 14 at the rates of 224 kg/ha for the field pea monocultures (FP), 123 kg/ha for oat monocultures (O), and 112 kg/ha for field pea and 67 kg/ha for oats in mixed plots (FP/O). All legumes were inoculated with commercial inoculum. We controlled weeds in the subplots by hand-weeding.

Due to farmer management inconsistencies, some data were omitted from analysis. The RC treatment was excluded from analysis for Field L6 because more than 1 replicate was compromised due to accidental mowing shortly before sampling. All annual plots for Field M3 were excluded from analysis due to dense clover growth in plots prior to planting. Farmers applied no fertilizers or amendments to the plots during the experiment with two exceptions. Composted poultry manure was spread on L2 at the rate of 3.4 t/ha and composted dairy manure was spread on H3 at an unknown rate in October 2006. We did not detect a strong response in plant variables to these late additions and data were retained for analysis with a few exceptions. Grass N uptake in Field L2 was highly variable, likely due to the influence of manure N uptake shortly before sampling, and these plots were omitted from analysis when they caused non-normal distribution of model residual errors.

Plant sampling and analysis

We sampled 0.25 m^2 of aboveground biomass from all subplots, avoiding plot edges, between October 8 and October 26, 2006. We separated legumes and grass biomass in mixed plots. If weeds were present, we measured weed weights for subplots. Weed biomass never amounted to more than 9% of total biomass and was not included in biomass estimates for analysis. Biomass was dried at 60° C and first coarsely ground using both a hammer mill and grinder and then finely pulverized using a roller grinder. Samples were analyzed for ^{15}N natural abundance and total N content using a continuous flow Isotope Ratio Mass Spectrometer (Stable Isotope Facility, UC Davis).

The Land Equivalency Ratio (LER) was calculated for annual and perennial mixed plots as a measure of the facilitative benefit of intercropping. The LER represents the land area required to achieve the same biomass yield as single crops. An LER greater than 1.0 suggests that the intercrop confers an advantage due to stronger effects of interspecific facilitation than competition (Vandermeer 1989). To answer the question of whether intercrops increased total biomass N accumulation relative to monocultures, we calculated LER on the basis of biomass nitrogen (N LER). The N LER was calculated as follows:

$$\text{N LER} = (N_{LM}/ N_L) + (N_{GM}/N_G)$$

where N_{LM} and N_L are the dry matter N content of legumes grown in mixture and monoculture, and N_{GM} and N_G are the dry matter N content of grasses grown in mixture and monoculture, respectively.

Nitrogen fixation

Nitrogen fixation was calculated using the ^{15}N natural abundance method (Shearer and Kohl 1986). The proportion of N derived from atmosphere in legume biomass (% N fixed) was calculated using orchardgrass as the reference plant for the red clover and oats as a reference plant for the peas:

$$\%\ \text{N fixed} = 100*((\ \delta^{15}\text{N Grass} - \delta^{15}\text{N legume})/(\ \delta^{15}\text{N Grass} - B))$$

where B is the $\delta^{15}N$ value of the legume grown with atmospheric N_2 as the only source of N after accounting for seed N. Total N fixed was calculated from the aboveground dry matter of the legume and its biomass N concentration and % N fixed.

To quantify the *B* value for red clover and field pea, we grew each legume in N-free, autoclaved, calcined clay media (Turface©, AIMCOR, Deerfield, IL) in a greenhouse. We surface sterilized seeds in 70% (v/v) ethanol for 3 minutes and 3%

(v/v) bleach solution for 2 minutes, followed by a 3 minute rinse in deionized water. We applied the same inoculant as used in field plots to sterilized seeds and fertilized plants with an N-free Hoagland's nutrient solution (GreenCare Fertilizers, Chicago, IL). We sampled plants at 9 weeks after planting for peas and 17 weeks after planting for clover, dried, ground, and analyzed for δ^{15}N. Seeds for peas, due to their larger size, were also analyzed for δ^{15}N to allow calculation of seed N contribution. The resulting *B* values used for calculations were -1.65 for red clover and -1.55 for field pea.

An estimate of transferred fixed N from legume to grass in mixed plots was calculated in a similar fashion, by comparing the δ^{15}N of the grass in the mixture to that of the grass grown in monoculture:

% grass N from transfer = 100*((δ^{15}N grass monoculture − δ^{15}N grass in mix)/(δ^{15}N grass monoculture − *C*))

where *C* is the δ^{15}N value of the integrated fractionation of δ^{15}N during N fixation in legume roots and between roots and sampled shoots of grass. For our purposes, *C* was set to the lowest δ^{15}N value of grass grown in mixture or zero, whichever was lowest. For oat, *C* was zero because all oat δ^{15}N values were positive and for orchardgrass, *C* was 1.37‰.

Statistical analysis

We computed statistics using JMP v.7 and SAS v.9.1 software (SAS Institute Inc., Cary, NC). Treatment means were compared using least squares means from mixed models including field and block as nested random factors. Treatment was nested within phenology type to reflect the different management histories of the annual and perennial plots. Multiple comparisons were calculated using Tukey's HSD and pairwise comparisons were calculated using Student's *t*-tests. To compare in-field variability for each treatment, coefficients of variance (CV) were calculated and compared using an analysis of variance (ANOVA). The % CV was calculated as the standard deviation divided by the sample mean, multiplied by 100. Correlations presented represent Pearson correlations of field means.

Results

Aboveground biomass and total N accumulation differed between mixtures and monocultures. The perennial mixture (RC/OG) produced more aboveground biomass than its constituent monocultures; however, the total N in aboveground biomass was similar for the perennial mixture and legume monoculture due to the lower average N concentration of mixture biomass (Table 3.3). The annual mixture (P/O) did not yield more than its constituent monocultures and the annual legume monoculture accumulated more total N in aboveground biomass than the annual mixture. All of the annual treatments assimilated a similar amount of soil N (Table 3.3). The perennial mixture assimilated more soil N than either perennial monoculture (Table 3.3).

Perennials accumulated more total biomass than annuals, but annuals had a faster growth rate (Table 3.3). The average growth period for the perennials was almost 3 times the growth period for the annuals (163 days and 63 days, respectively). The earlier seeding date for the perennial was likely the primary cause of its greater biomass accumulation and total N fixed compared to the fall-seeded annual field pea (Table 3.3). Controlling for the differences in growth periods, the average biomass accumulation rate for the annuals was greater compared to the perennials (22 and 16 kg ha^{-1} d^{-1}, respectively).

The complementary interactions of grasses and legumes in mixtures conferred an advantage in total N mobilization when compared with corresponding

Table 3.3. Least squares means for aboveground plant biomass and nitrogen variables for 6 plant treatments across 15 farm fields. O = oats; FP = field pea monoculture; FP/O = field pea-oat mixture, OG = orchardgrass; RC = red clover monoculture; RC/OG = red clover-orchardgrass mixture. Different letters within a column represent significant differences between plant treatments ($p<0.05$).

Plant treatment	**Above-ground biomass**	**Legume biomass**	**Grass biomass**	**Total plant N**	**Soil N uptake**	**Legume soil N uptake**	**Grass soil N uptake**	**N fixed**
	kg ha^{-1}							
O	1104 d		1104 b	20 d	20 c		20 b	
FP	1627 c	1627 b		55 b	18 c	18 b		37 b
FP/O	1417 cd	690 c	747 c	37 c	20 c	4 d	16 b	17 c
OG	2221 b		2221 a	32 c	32 b		32 a	
RC	2364 b	2364 a		75 a	27 b	27 a		48 a
RC/OG	2894 a	1522 b	1371 b	74 a	41 a	13 c	29 a	33 b

monocultures. The average N LER for perennial and annual mixtures was 1.6 and 1.2, respectively. The N LER for perennials was equal to or greater than 1 for all fields, while 3 fields had an N LER less than 1 for the annual (Figure 3.1).

Competitive plant species interactions influenced both legume reliance on N fixation and total N fixed for both the annual and perennial legume in mixtures. The relative reliance on N fixation (% N from fixation) was greater for both mixtures than their respective monocultures (Figure 3.2). The average % N from fixation for mixtures and monocultures was 72% and 64% for perennial clover, and 80% and 68% for annual pea, respectively. Legume biomass as a proportion of total mixture biomass was more variable than the % N from fixation and ranged from 26% to 99% and from 18% to 84% for the perennial and annual, respectively (Figure 3.3). Due to the lower legume biomass in the mixtures compared with monoculture plots, the total N fixed was lower in mixtures than monocultures (Table 3.3).

We found evidence of the facilitative transfer of N from legumes to grasses in mixed plots. Estimates of N transfer from legumes to grasses in mixed plots were greater for perennials compared to annuals. The average δ^{15}N signature of orchardgrass grown in mixtures was significantly less enriched than orchardgrass in monocultures (Figure 3.4). There was no significant difference in the average δ^{15}N signature of oats grown in mixtures or monocultures. Using this shift in δ^{15}N signatures to estimate the proportion of grass N derived from legume N fixation, N transfer was positively correlated with the relative abundance of legumes in mixed plots (Figure 3.5). Using the δ^{15}N abundance method, field average estimates of the amount of fixed N transferred to orchardgrass in RC/OG ranged as high as 17 ± 2 kg N ha^{-1}. If the average fixed N transferred of 5 ± 1 kg N ha^{-1} was added to the average of 33 kg N ha^{-1} fixed by clover in mixed plots, this would add an additional 15% to N

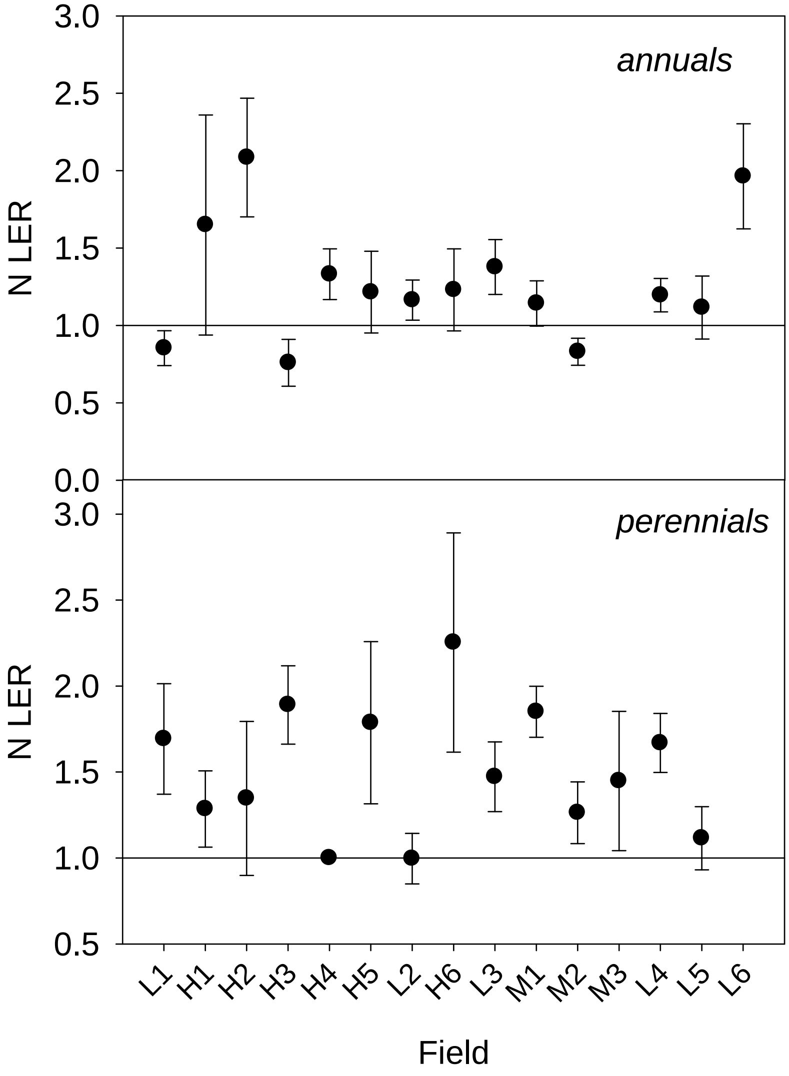


Figure 3.1. Field average N Land Equivalence Ratio (N LER) for perennial red clover/orchardrass and annual field pea/oat mixtures. Fields are sorted by increasing soil N availability as defined by PC 2 from principal components analysis. Error bars represent standard errors (n=4).

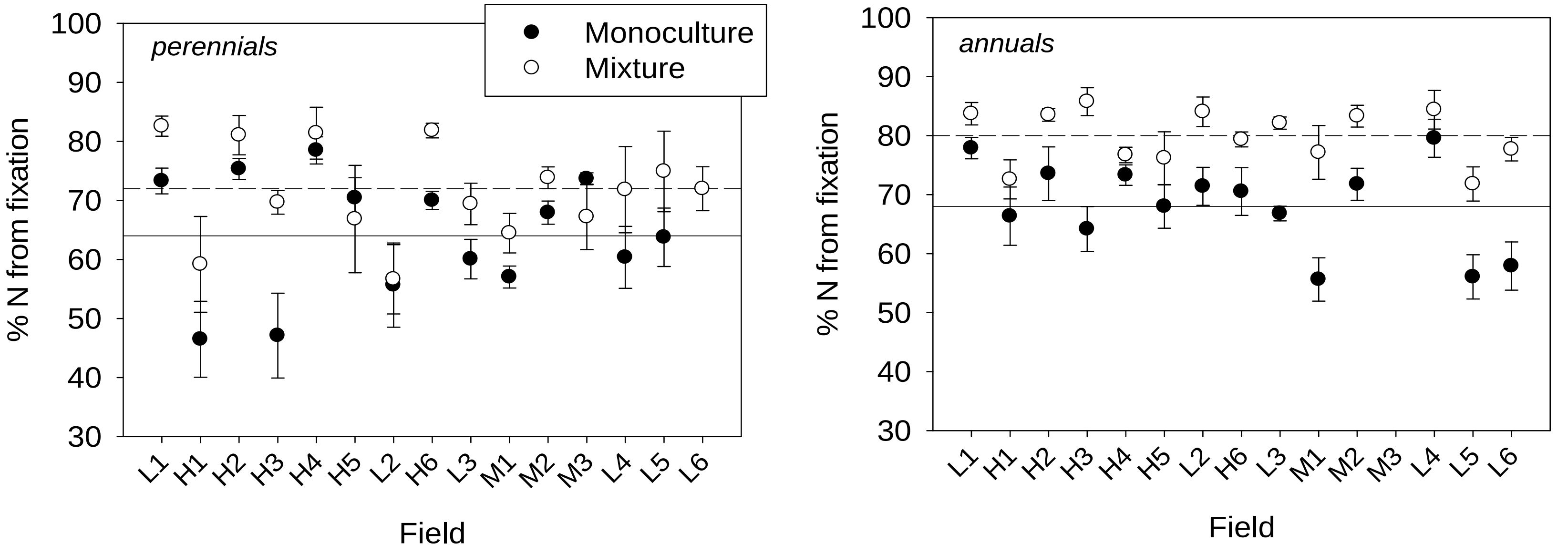


Figure 3.2. Field average % N from fixation of perennial red clover and annual field pea grown in monoculture or in mixture with a grass. Horizontal lines represent overall means for legumes in monoculture (solid line) and mixtures (dashed line). Fields are sorted by increasing soil N availability as defined by PC 2 from principal components analysis. Error bars represent standard errors (n=4).

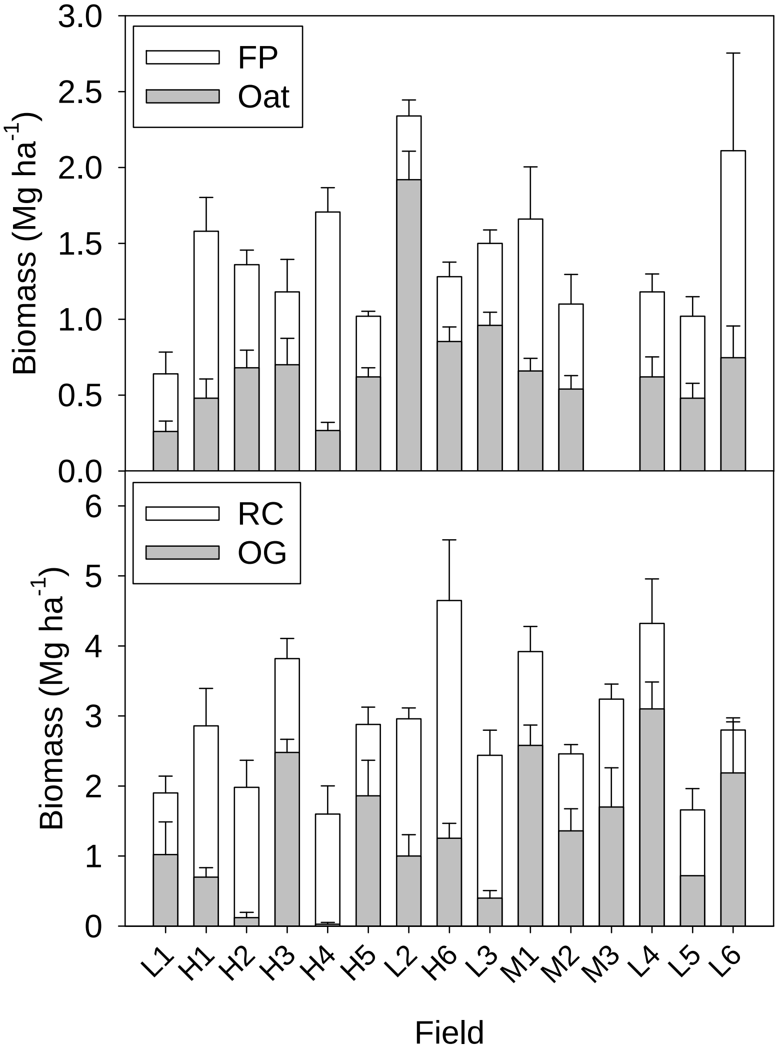


Figure 3.3. Grass and legume aboveground biomass in mixtures across fields. FP= field pea; RC= red clover; OG= orchardgrass. Fields are sorted by increasing soil N availability as defined by PC 2 from principal components analysis. Error bars represent standard errors (n=4)

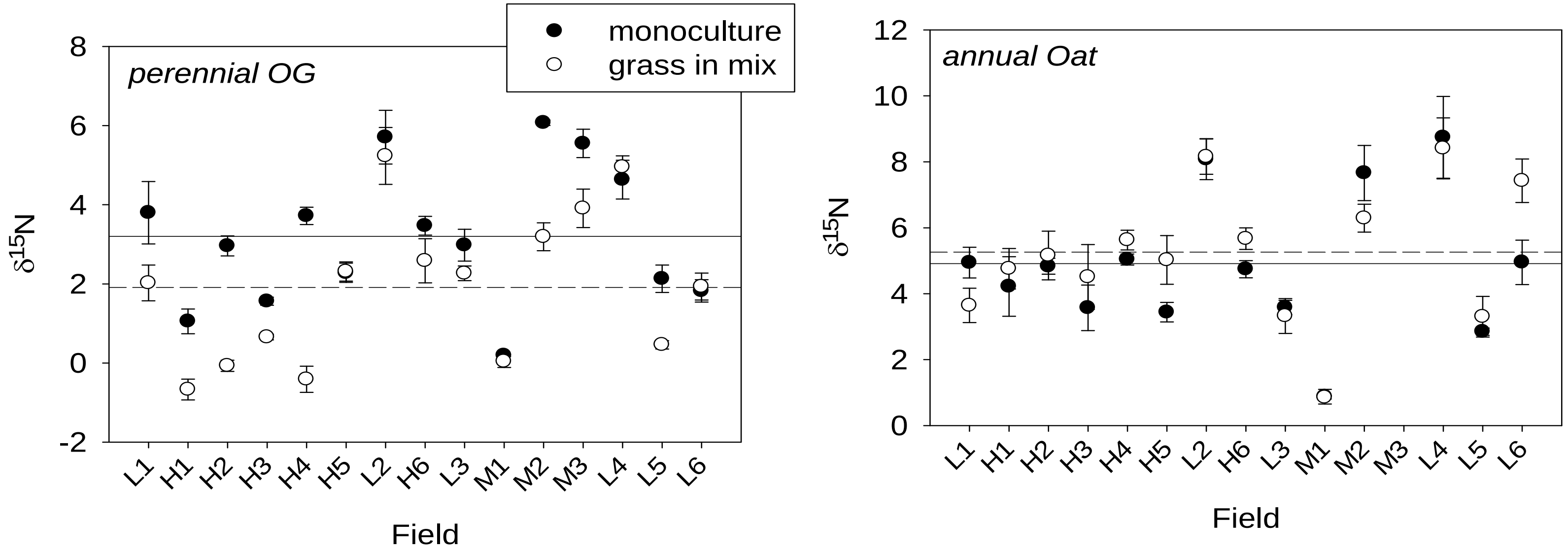


Figure 3.4. Field average δ^{15}N for grasses grown in monoculture or in legume mixture. Horizontal lines represent overall means for monoculture (solid line) and mixtures (dashed line). Fields are sorted by increasing soil N availability as defined by PC 2 from principal components analysis. Error bars represent standard errors (n=4).

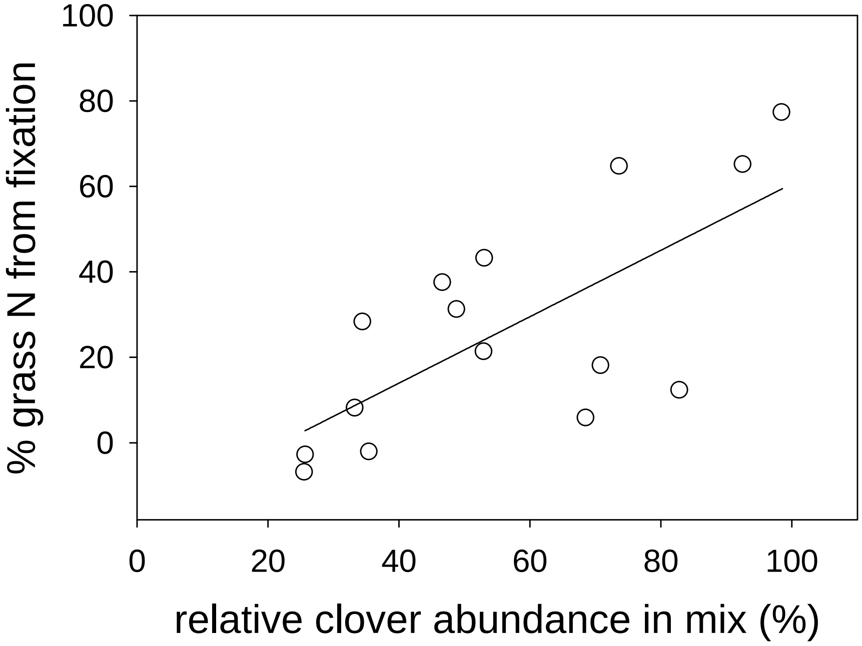


Figure 3.5. Correlation between the relative abundance of red clover in clover-orchardgrass mixtures and the estimated percentage of orchardgrass N derived from red clover fixed N increased (% grass N from fixation).

fixation estimates.

Plant N concentrations also provided evidence of increased N availability to grasses in both the perennial and annual mixtures. Grass N concentrations were significantly higher in mixed plots than in monocultures and the difference was greater for OG than O (Figure 3.6). In contrast, legume N concentration was lower in mixtures than in monoculture plots (Figure 3.6).

Total biomass accumulation was strongly correlated with total N fixed in all treatments except for the perennial monoculture. Both soil N assimilation and the amount of N fixed were strongly positively correlated with total biomass for FP, FP/O, and RC/OG (Figure 3.7). Soil N assimilation was positively correlated with biomass for RC, but total N fixed was not (Figure 3.7). The % N from fixation was positively correlated with N fixed for RC ($r^2 = 0.55$, $p=0.003$), but not for the other treatments ($p>0.05$).

Particularly striking was the relative stability of RC biomass across the 15 fields compared to the other plant treatments. The coefficients of variation (CV) for RC biomass N and N fixed were less than half of the CVs for the other treatments (Figure 3.8). In contrast, the variance for the % N from fixation across sites was greater for RC than the other treatments (Figure 3.8). Total aboveground biomass variability was similar for all treatments, except RC, both within and across fields.

Mixtures had higher within and across field variability in N fixed than monocultures. The total N fixed in mixtures was highly variable across fields (Figure 3.8). The variability in N fixed was driven primarily by the relative abundance of legumes within mixtures ($r=0.78$, $p=0.0006$). The % of N from fixation was not significantly correlated with total N fixed in mixtures ($p>0.05$).

Principal component analysis was used to investigate the effects of soil fertility

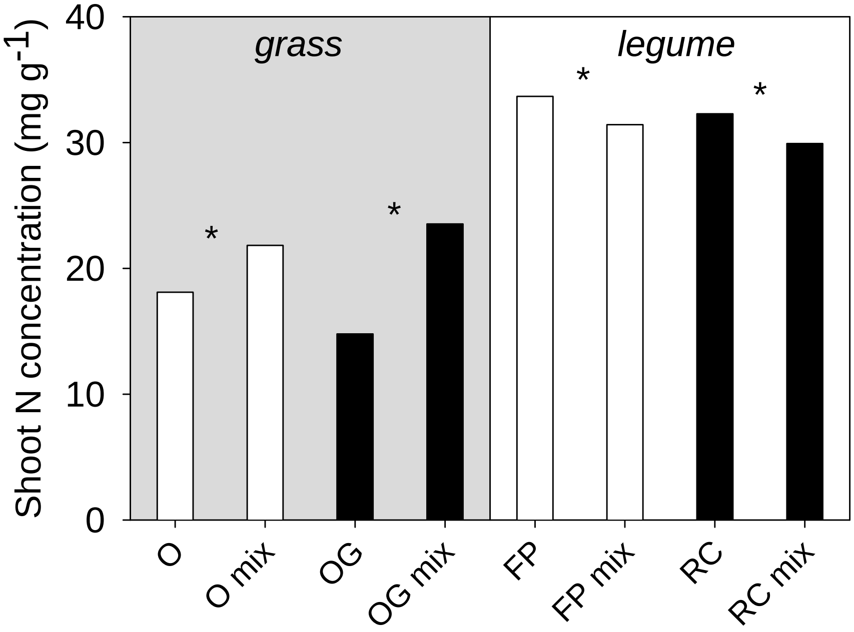


Figure 3.6. Comparison of average shoot N concentration between mixed and monoculture plots of annual (open bars) and perennial (filled bars) grasses and legumes. Stars indicate significant pair-wise differences ($p<0.05$). O= oats; OG= orchardgrass; FP= field pea; RC= red clover

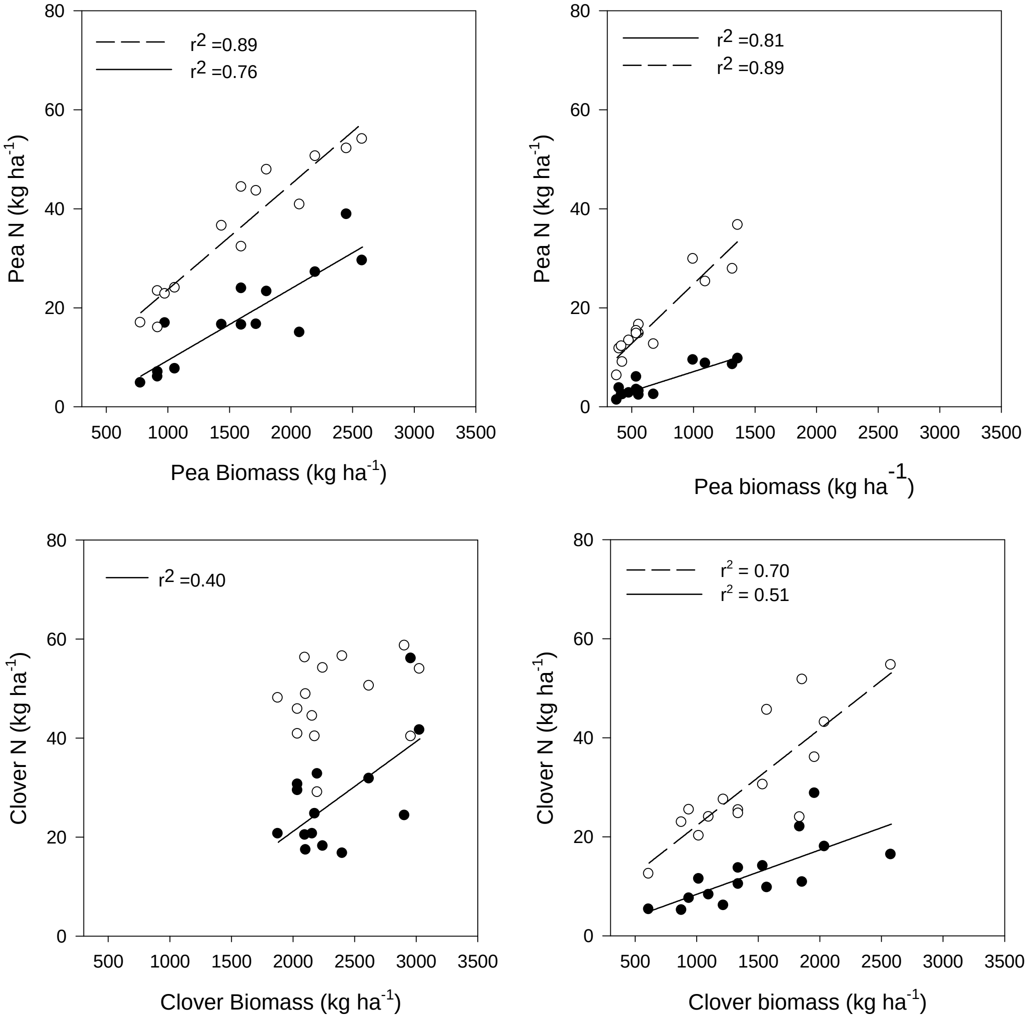


Figure 3.7. Relationships between total legume aboveground biomass, N from fixation (open circles), and N from soil (filled circles) for pea in monoculture (FP), pea in mixture with oats (FP/O), red clover in monoculture (RC), and red clover in mixture with orchardgrass (RC/OG). Scale is constant across graphs to emphasize differences in variability across treatments.

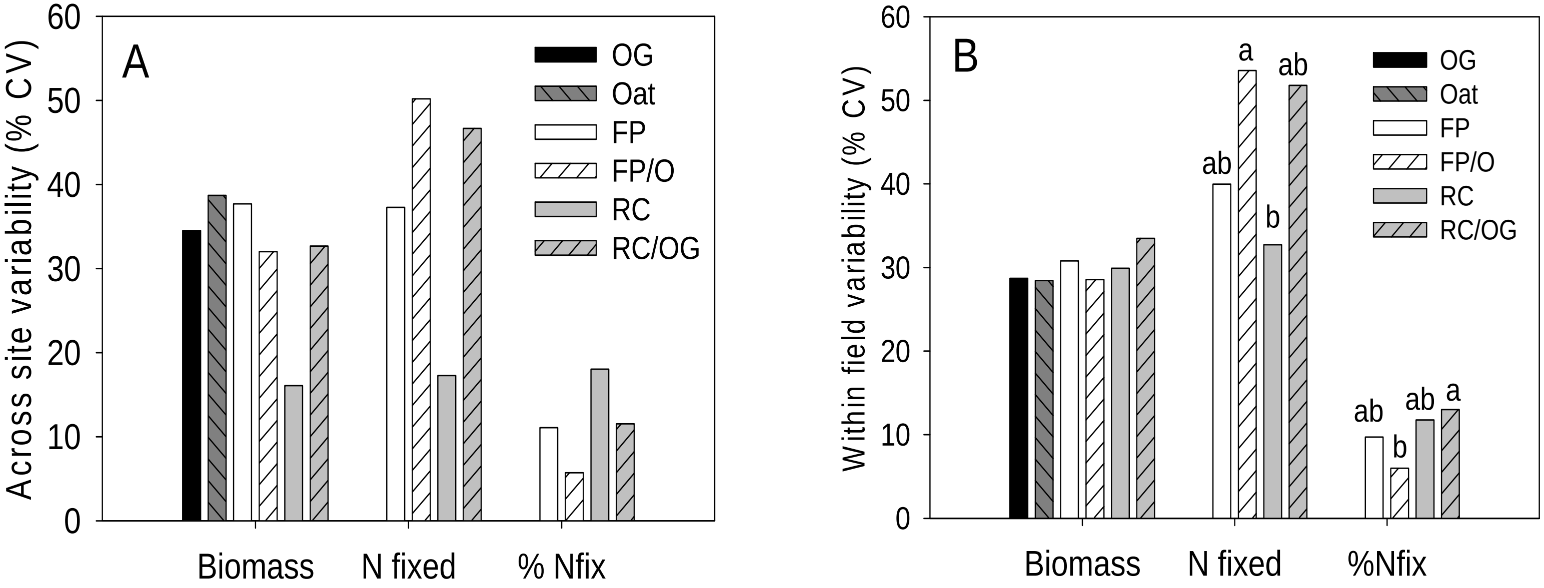


Figure 3.8. Coefficients of variation (% CV) for total aboveground N, N fixed, and % N from fixation (% Nfix) for all six plant treatments a) across the 15 fields; and b) within fields. Different letters above bars represent significant differences between plant treatment in-field variability ($p<0.05$). Plant treatmentds include orchardgrass (OG), oats (O), field pea in monoculture (FP), field pea in mixture with oats (FP/O), red clover in monoculture (RC), and red clover in mixture with orchardgrass (RC/OG).

and texture on N fixation. Labile N availability was represented by PC2 (Table 3.2) and explained a significant proportion of the variability in soil N uptake for both grass treatments. Oat and OG soil N uptake were correlated with PC2 (Table 3.4). Soil N uptake for oat grown in mixture was also positively correlated with PC2. The positive relationships between PC2 and the grass treatments indicate that there was an N-based fertility gradient across the 15 fields. Grass N assimilation, one potential indicator of soil N availability, ranged from 12 to 84 kg N ha^{-1} for orchardgrass and from 7 to 49 kg N ha^{-1} for oats among field sites (data not shown).

Evidence of soil N availability inhibiting N fixation was limited. Monoculture field pea % N from fixation had the strongest negative correlation with PC2 of any of the legumes ($p=0.15$; Table 3.4). Pairwise correlations with individual soil N variables revealed that the % N from fixation for field pea was negatively correlated with microbial biomass N ($r= -0.59$, $p=0.03$). Soil N availability may have indirectly reduced N fixation of clover in mixed plots by supporting increased orchardgrass biomass, thereby, suppressing clover biomass. The biomass of red clover in mixtures relative to total RC/OG biomass was negatively correlated with June soil nitrate measurements ($r= -0.52$, $p=0.05$) and, correspondingly, was weakly correlated with PC2 ($p=0.08$; Table 3.4).

Red clover growth and N fixation were influenced by soil texture. Texture, represented by PC1, was correlated with RC % N from fixation indicating increased reliance on N fixation in finer textured soils. The negative correlation between PC1 and OG biomass in mixtures may be a result of increased RC competition in fields with finer textured soils. Texture effects on red clover were also reflected by a significant correlation between PC1 and the relative abundance of legume biomass in RC/OG mixtures (Table 3.4).

Table 3.4. Pearson correlations between principal components and aboveground biomass, soil N uptake, the % N from fixation, and the relative abundance of legumes in mixtures for oats (O), orchardgrass (OG), field pea in monoculture (FP), field pea in mixture with oats (FP/O), red clover in monoculture (RC), and red clover in mixture with orchardgrass (RC/OG). PC1 represents soil texure and total organic matter, PC2 represents soil N availability, and PC3 represents soil P and K availability.

	Biomass			Soil N uptake			% N from fixation			Leg biomass as % of total		
Treatment	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
O	-0.30	0.46	0.19	-0.23	**0.65***	0.14						
OG	-0.05	**0.55***	0.16	-0.02	**0.60***	-0.04						
FP	-0.26	-0.18	**0.67****	-0.32	0.10	0.52	0.03	-0.41	-0.18			
FP in mix	0.43	-0.20	0.32	0.22	-0.02	0.30	-0.21	-0.02	0.01			
O in mix	0.05	0.39	0.12	0.00	**0.57***	0.08						
FP/O total	0.47	0.20	0.43	0.09	**0.57***	0.23	-0.26	-0.07	-0.01	0.31	-0.37	0.06
RC	0.18	-0.33	0.18	-0.47	-0.06	0.26	**0.54***	-0.04	-0.43			
RC in mix	0.32	-0.31	0.41	0.06	-0.26	0.27	0.25	0.01	-0.29			
OG in mix	**-0.59***	0.42	0.24	**-0.57***	0.39	0.37						
RC/OG total	-0.37	0.21	**0.57***	-0.50	0.34	**0.53***	0.31	-0.26	-0.43	0.62*	-0.48	0.08

* $p<0.05$, **$p<0.01$

The availability of other soil nutrients may have limited growth of both annual and perennial legumes. PC3, for which P and K had the strongest loadings, explained a significant percentage of the variation in field pea biomass and RC/OG biomass and soil N uptake, suggesting that P and K availability may have limited biomass accumulation for these treatments (Table 3.4).

Discussion

Complementary and competitive interactions in mixtures

Species mixtures can improve total nutrient cycling efficiency due to complementary nutrient acquisition in space and time (Hooper and Vitousek 1998; Vandermeer 1989). We found evidence of complementary resource use in mixtures, reflected by the greater biomass N accumulation of grass-legume mixtures than corresponding monocultures. The average N LER values for mixtures were greater than 1.0 for both annual and perennial mixtures (Figure 3.1). The evidence for complementary resource use was stronger for perennials than annuals. Perennial mixtures had higher N LER values, total biomass, and soil N uptake than either corresponding monoculture. In contrast, total biomass and soil N uptake of monoculture field pea were equivalent to the field pea/oat mixture (Table 3.3). The complementarity of the species in the perennial mixture may be due to niche differentiation resulting from differences in root architecture and phenology (Malezieux et al. 2009). Red clover is a tap-rooted species while orchardgrass has a more fibrous root system typical of grass species. Fornara and Tilman (2008) found that the complementarity of grass-legume resource use in grasslands increased total biomass, particularly root biomass, resulting in greater soil C and N accumulation relative to corresponding monocultures.

Complementarity in species mixtures can also result from facilitative interactions (Brooker et al. 2008). We found evidence for facilitative N transfer from legumes to grasses in perennial mixtures, but not in annual mixtures. Based on shifts in $\delta^{15}N$ of grasses grown in mixture and monoculture, relative contributions of fixed N to perennial orchardgrass N ranged from -7% to 77% (Figure 3.5). Moyer-Henry et al. (2006) found a similar range of N transfer rates from soybean to associated weed species using the ^{15}N natural abundance method. However, due to the spatial variability in soil ^{15}N natural abundance, small differences in ^{15}N natural abundance alone do not provide conclusive evidence of N transfer (Walley et al. 2001). Rooting patterns can also shift when grasses are grown in mixtures, which could result in N uptake from different soil N pools of grasses in monoculture and mixtures (Hauggaard-Nielsen and Jensen 2001). Higher $\delta^{15}N$ values for grasses in mixtures than monocultures for some fields, particularly for annual grasses, indicate the uncertainty of transfer estimates based on ^{15}N natural abundance methods alone. In addition, fixed N in grasses can also be derived from associative N fixation (Reis et al. 2000).

We found two forms of supporting evidence for the transfer of N from legumes to grasses. The correlation between shifts in $\delta^{15}N$ values and the relative abundance of red clover in perennial mixtures, and the increased concentration of N in grasses in mixtures confirm that legumes improved grass N accumulation (Figures 3.5 and 3.6). In addition, similarly high levels of N transfer in red clover-orchardgrass mixtures were also measured by Farnham and George (1993) using the ^{15}N isotope dilution method.

While facilitative interactions contributed to increased total grass N assimilation and may have supported greater biomass production in grass-legume mixtures, competition for soil N and light availability influenced N fixation inputs.

The higher reliance on N fixation in mixtures than monocultures is in agreement with previous studies (Carlsson and Huss-Danell 2003). Legume N fixation rates are increased in mixtures because grasses tend to outcompete legumes for available soil N (Munoz and Weaver 1999). While the average % N from fixation across sites was higher in mixtures than monocultures, values were not statistically different in several fields for both annuals and perennials (Figure 3.2). Total legume N fixation in mixtures was strongly correlated with aboveground legume biomass. The species composition in mixtures shifted across fields (Figure 3.3), and was likely caused by the interaction of differential species responses to soil resources and competition for light availability.

Phenological differences

Facilitative species interactions require time to develop. The greater N transfer from legumes to grasses in perennial mixtures than annual mixtures is likely due to differences in growth period. Evidence of N transfer in annual pea-grain intercrops has been inconsistent, while consistently higher levels of N transfer have been measured in perennial grass-legume pastures (Hogh-Jensen and Schjoerring 2000; Jensen 1996). Nitrogen transfer can occur directly through mycorrhizal hyphae and indirectly through legume root turnover and N-rich root exudates (Paynel and Cliquet 2003; Ta and Faris 1987). The short growth period of the annual mixture (63 days) was too short for fine root turnover, in particular, to play a significant role in N transfer (Goins and Russelle 1996).

Including estimates of root biomass and N rhizodeposition would accentuate the differences between annuals and perennials. Perennials develop more extensive root systems than most annuals (Schenk and Jackson 2002), which can confer an advantage to accessing soil N in a heterogeneous soil environment (Casper and

Jackson 1997) and reducing N leaching from agroecosystems (Crews 2005). Hogh-Jensen and Schjoerring (2001) measured approximately 60% of total red clover N in belowground roots and rhizodeposits within the first year. Using similar methods, field pea belowground N represented less than 20% of total plant N (Mayer et al. 2003).

Direct comparisons between annual and perennial % N from fixation are problematic due to the high sensitivity of the ^{15}N natural abundance method to the reference plant used (Pate et al. 1994). Oat δ^{15}N signatures were consistently higher than orchardgrass δ^{15}N (Figure 3.4). This could be due to a combination of temporal differences in the δ^{15}N of available soil N and differences in rooting patterns resulting in the acquisition of soil N from different soil depths and pools. The relative shifts in N fixation of the two legumes, however, are robust measurements due to the consistent use of the same reference species across all sites.

Soil effects on BNF and species interactions

The accepted concept that soil N availability is a dominant edaphic factor influencing legume N fixation has rarely been tested across a gradient of N availability driven by the mineralization of endogenous SOM pools. We found limited evidence of N fixation inhibition by soil N availability. Annual field pea monoculture % N from fixation had the strongest negative correlation with soil N availability. The lack of any correlation between the % N from fixation and soil N availability for the other treatments is consistent and in contrast to the response of grass N uptake (Table 3.4). This suggests that legume N fixation alleviated N limitation, but N availability was insufficient to suppress BNF. Most research has focused on the response of N fixation to recent pulses of labile N additions through HBN fertilizer applications at different rates (e.g., Boller and Nosberger 1994; Hogh-Jensen and Schjoerring 1997).

Our results suggest that N fixation is not sensitive to the range of soil N availability driven by mineralization of organic matter levels included in this study. Soil organic matter levels varied more than 2-fold across field sites and soil N assimilation of grasses varied more than 6-fold. Monoculture orchardgrass N assimilation in more fertile fields was as high as 84 kg N ha^{-1} in a year when no fertilizers were added. The lack of a relationship between BNF estimates and soil N availability is likely because nitrogen mineralization from heterogeneous SOM pools created temporally variable microsite hotspots of inorganic N throughout the rooting zone. This is in contrast to the potential influence of the broad application of HBN fertilizers at rates that typically saturate the root system for a short, concentrated time period.

The low variability in NPP of the perennial monoculture among fields compared to all other plant treatments was unexpected. A central tenet of ecological theory is that increasing biodiversity increases the stability of ecosystem properties, such as NPP (Hooper et al. 2005). Increasing species diversity confers greater stability of ecosystem properties because species composition can shift in response to environmental changes, buffering the different sensitivities of species to environmental change (Frank and McNaughton 1991). While legume-grass mixtures out-yielded corresponding monocultures, mixtures did not have greater NPP stability across field sites in this study (Figure 3.8). The stability of clover NPP across sites suggests that in low fertility sites it possessed the plasticity to maintain productivity, while in more fertile sites it was less able to take advantage of greater resource availability, compared with the perennial grass-legume mixtures. Red clover has undergone less intensive breeding selection than commodity grain crops and the genetic diversity within individual cultivars is similar to the diversity between cultivars (Yu et al. 2001). Intra-cultivar diversity may have contributed to the stability of clover NPP in this study. Due to this stability in NPP, differences in the % N from fixation were more important in determining total N fixed than aboveground NPP in clover.

Species relative abundance in mixtures did shift across sites, but soil variables did not explain the variability in mixture composition. The total N fixed in mixtures was strongly correlated with legume biomass. The weak negative correlation between PC2 and the biomass of legumes relative to total mixture biomass for perennial mixtures suggests that soil N availability did not influence N fixation rates directly. Instead, greater soil N availability influenced N fixation indirectly by supporting greater biomass production by the grass, which in turn suppressed the total legume biomass produced. At high soil N availability, grasses tend to outcompete legumes for resources, including light and soil N (Nesheim and Oyen 1994).

Management implications

Plants can be utilized as tools for nutrient management through identification of species and species assemblages that serve different functional roles for integration at different times in crop rotations and at different stages of soil fertility development. For example, annual field pea provided a substantial input of fixed N within a short time window, while perennial grass-legume mixtures increased soil N uptake, which can be important for reducing losses of residual N fertilizers or in soils with high N mineralization rates. In addition, a challenge in legume management is the lack of synchrony between the rapid, initial decomposition of N-rich litter following incorporation and the N uptake of a subsequent crop (Crews and Peoples 2005). Grass-legume mixtures may improve synchrony through the slower decomposition of litter with higher C:N, resulting in short-term N immobilization and retention into microbial biomass and SOM pools.

Conclusions

Understanding the ecology of legume cover crops is critical for the selection of species and species assemblages to design sustainable agroecosystems. The effects of species interactions and plant phenology on ecosystem properties have received little attention in agricultural research due to the dominance of annual, monocrop systems (Malezieux et al. 2009). We found evidence for improved nutrient use efficiency and yield stability in perennial mixtures and monocultures. The lack of an influence of soil N availability on N fixation across the fields in this study illustrates the importance of testing relationships indentified under relatively controlled experimental conditions within the context of heterogeneous agroecosystems to understand their functional significance. These results support the need for more systems-based tools and research approaches for the development of sustainable, multispecies agroecosystems.

REFERENCES

Boller BC, Nosberger J (1994) Differences in nitrogen-fixation among field-grown red clover strains at different levels of ^{15}N fertilization. Euphytica 78:167-174

Brooker RW, Maestre FT, Callaway RM, Lortie CL, Cavieres LA, Kunstler G, Liancourt P, Tielborger K, Travis JMJ, Anthelme F, Armas C, Coll L, Corcket E, Delzon S, Forey E, Kikvidze Z, Olofsson J, Pugnaire F, Quiroz CL, Saccone P, Schiffers K, Seifan M, Touzard B, Michalet R (2008) Facilitation in plant communities: the past, the present, and the future. Journal Of Ecology 96:18-34

Carlsson G, Huss-Danell K (2003) Nitrogen fixation in perennial forage legumes in the field. Plant and Soil 253:353-372

Casper BB, Jackson RB (1997) Plant competition underground. Annual Review Of Ecology And Systematics 28:545-570

Crews TE (2005) Perennial crops and endogenous nutrient supplies. Renewable Agriculture and Food Systems 20:25-37

Crews TE, Peoples MB (2005) Can the synchrony of nitrogen supply and crop demand be improved in legume and fertilizer-based agroecosystems? A review. Nutrient Cycling in Agroecosystems 72:101-120

Drinkwater LE, Snapp SS (2007) Nutrients in agroecosystems: rethinking the management paradigm. Advances in Agronomy 92:163-186

Elgersma A, Schlepers H, Nassiri M (2000) Interactions between perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) under contrasting nitrogen availability: productivity, seasonal patterns of species composition, N_2 fixation, N transfer and N recovery. Plant and Soil 221:281-299

Farnham DE, George JR (1993) Dinitrogen fixation and nitrogen transfer among red clover cultivars. Canadian Journal of Plant Science 73:1047-1054

Fornara DA, Tilman D (2008) Plant functional composition influences rates of soil carbon and nitrogen accumulation. Journal of Ecology 96:314-322

Frank DA, McNaughton SJ (1991) Stability increases with diversity in plant-communities - empirical-evidence from the 1988 Yellowstone drought. Oikos 62:360-362

Galloway JN, Cowling EB (2002) Reactive nitrogen and the world: 200 years of change. Ambio 31:64-71

Goins GD, Russelle MP (1996) Fine root demography in alfalfa (Medicago sativa L). Plant and Soil 185:281-291

Hardarson G, Zapata F, Danso SKA (1984) Effect of plant genotype and nitrogen-fertilizer on symbiotic nitrogen-fixation by soybean cultivars. Plant and Soil 82:397-405

Hauggaard-Nielsen H, Jensen ES (2001) Evaluating pea and barley cultivars for complementarity in intercropping at different levels of soil N availability. Field Crops Research 72:185-196

Hogh-Jensen H, Schjoerring JK (1997) Interactions between white clover and ryegrass under contrasting nitrogen availability: N-2 fixation, N fertilizer recovery, N transfer and water use efficiency. Plant and Soil 197:187-199

Hogh-Jensen H, Schjoerring JK (2000a) Below-ground nitrogen transfer between different grassland species: direct quantification by ^{15}N leaf feeding compared with indirect dilution of soil ^{15}N. Plant and Soil 227:171-183

Hogh-Jensen H, Schjoerring JK (2000b) Below-ground nitrogen transfer between different grassland species: Direct quantification by N-15 leaf feeding compared with indirect dilution of soil N-15. Plant and Soil 227:171-183

Hogh-Jensen H, Schjoerring JK (2001) Rhizodeposition of nitrogen by red clover, white clover and ryegrass leys. Soil Biology & Biochemistry 33:439-448

Hooper DU, Chapin FS, Ewel JJ, Hector A, Inchausti P, Lavorel S, Lawton JH, Lodge DM, Loreau M, Naeem S, Schmid B, Setala H, Symstad AJ, Vandermeer J, Wardle DA (2005) Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. Ecological Monographs 75:3-35

Hooper DU, Vitousek PM (1998) Effects of plant composition and diversity on nutrient cycling. Ecological Monographs 68:121-149

Jensen ES (1996) Barley uptake of N deposited in the rhizosphere of associated field pea. Soil Biology & Biochemistry 28:159-168

Kiers ET, Hutton MG, Denison RF (2007) Human selection and the relaxation of legume defences against ineffective rhizobia. Proceedings of the Royal Society B-Biological Sciences 274:3119-3126

Lamb JFS, Barnes DK, Russelle MP, Vance CP, Heichel GH, Henjum KI (1995) Ineffectively and effectively nodulated alfalfas demonstrate biological nitrogen-fixation continues with high-nitrogen fertilization. Crop Science 35:153-157

Malezieux E, Crozat Y, Dupraz C, Laurans M, Makowski D, Ozier-Lafontaine H, Rapidel B, de Tourdonnet S, Valantin-Morison M (2009) Mixing plant species in cropping systems: concepts, tools and models. A review. Agronomy For Sustainable Development 29:43-62

Mayer J, Buegger F, Jensen ES, Schloter M, Hess J (2003) Estimating N rhizodeposition of grain legumes using a N-15 in situ stem labelling method. Soil Biology & Biochemistry 35:21-28

McIsaac GF, David MB, Gertner GZ, Goolsby DA (2001) Eutrophication - Nitrate flux in the Mississippi River. Nature 414:166-167

Mitsch WJ, Day JW, Gilliam JW, Groffman PM, Hey DL, Randall GW, Wang NM

(2001) Reducing nitrogen loading to the Gulf of Mexico from the Mississippi River Basin: Strategies to counter a persistent ecological problem. Bioscience 51:373-388

Moyer-Henry KA, Burton JW, Israel DW, Rufty TW (2006) Nitrogen transfer between plants: A 15N natural abundance study with crop and weed species. Plant and Soil 282:7-20

Munoz AE, Weaver RW (1999) Competition between subterranean clover and rygrass for uptake of N-15-labeled fertilizer. Plant And Soil 211:173-178

Nesheim L, Oyen J (1994) Nitrogen fixation by red clover (*Trifolium pratense* L) grown in mixtures with timothy (*Phleum pratense* L) at different levels of nitrogen fertilization. Acta Agriculturae Scandinavica Section B-Soil and Plant Science 44:28-34

Pate JS, Unkovich MJ, Armstrong EL, Sanford P (1994) Selection of reference plants for ^{15}N natural abundance assessment of N_2 fixation by crop and pasture legumes in South-West Australia. Australian Journal of Agricultural Research 45:133-147

Paynel F, Cliquet JB (2003) N transfer from white clover to perennial ryegrass, via exudation of nitrogenous compounds. Agronomie 23:503-510

Ranells NN, Wagger MG (1997) Grass-legume bicultures as winter annual cover crops. Agronomy Journal 89:659-665

Reis VM, Baldani JI, Baldani VLD, Dobereiner J (2000) Biological dinitrogen fixation in gramineae and palm trees. Critical Reviews In Plant Sciences 19:227-247

Schenk HJ, Jackson RB (2002) Rooting depths, lateral root spreads and below-ground/above-ground allometries of plants in water-limited ecosystems. Journal Of Ecology 90:480-494

Shearer G, Kohl DH (1986) N_2 fixation in field settings: estimates based on natural ^{15}N abundance. Australian Journal of Plant Physiology 13:699-756

Ta TC, Faris MA (1987) Species variation in the fixation and transfer of nitrogen from legumes to associated grasses. Plant and Soil 98:265-274

Tonitto C, David MB, Drinkwater LE (2006) Replacing bare fallows with cover crops in fertilizer-intensive cropping systems: A meta-analysis of crop yield and N dynamics. Agriculture Ecosystems & Environment 112:58-72

Vandermeer J (1989) The ecology of intercropping. Cambridge University Press, Cambridge

Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm NB, Howarth RW, Marino R, Martinelli L, Rastetter EB, Sprent JI (2002) Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 57/58:1-45

Walley F, Fu GM, van Groenigen JW, van Kessel C (2001) Short-range spatial variability of nitrogen fixation by field-grown chickpea. Soil Science Society of America Journal 65:1717-1722

Woodmansee R (1984) Comparative nutrient cycles of natural and agricultural ecosystems: a step toward principles. In: Lowrance BR, Stinner R, House GJ (eds) Agricultural ecosystems: unifying concepts. J. Wiley, New York, pp 145-156

Yu J, Mosjidis JA, Klingler KA, Woods FM (2001) Isozyme diversity in North American cultivated red clover. Crop Science 41:1625-1628