Soybean nitrogen fixation dynamics in Iowa, USA

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Abstract

The rainfed US Midwestern region has deep, fertile soils and leads the US in soybean [*Glycine max*, (L.) Merr.] production. Biological nitrogen (N) fixation (BNF) contributes a portion of the soybean N requirement, but variability in BNF is poorly understood and estimates of BNF for this region are rare. We established experiments in Iowa, USA to gain a better understanding of BNF and increase its predictability. We collected in-season BNF measurements accompanied by high temporal resolution soil and plant growth measurements. Across two years, two locations and two planting dates, we found that BNF contributed 23-65% of total aboveground N accumulation in soybean. The BNF rate was maximized at the early seed-filling period and varied from 1 to 3 kg N $ha^{-1}day^{-1}$. During seed filling period, the rate of BNF was related to crop growth rate (carbon (C) supply) but not to N accumulation by the reproductive organs (N demand). We found that a minimum crop growth rate of 135 kg dry matter $ha^{-1}day^{-1}$ is required to sustain maximum BNF rates. In contrast to BNF, the soil inorganic N uptake rate was related to seed N demand but not to C supply. Biomass production was the best predictor of total soybean BNF ($R^2 > 0.83$). On average, 0.013 kg N was fixed per kg biomass produced. Across all trials, the N exported via seed was greater than the N imported via BNF, which suggests that Midwest US soybeans may reduce soil organic matter. We concluded that future research efforts should focus on increasing C – rather than N – availability during the seed filling period towards improving both grain yields and environmental sustainability.

Keywords: crop growth rates, soil nitrate, soil water, biomass, N balance

1 Introduction

Two sources of nitrogen (N) contribute to total soybean N uptake: biological N fixation (BNF) and soil inorganic N. There is tremendous variation in the amount of N that is derived from BNF versus the soil. According to Ciampitti and Salvagiotti (2018), BNF contributes on average 55% of the total N accumulated in aboveground biomass (range: 0–94%). . Uncertainty about BNF hinders decisions about N fertilizer management for soybean as well as the implementation of N management decision programs that aim to reduce environmental N losses (Christianson et al., 2012; McLellan et al., 2018).

Estimating and explaining variations in the amount and timing of BNF remains a challenge (Liu et al., 2011). Multiple factors contribute to the variability in BNF including water availability (Purcell et al. 2004), soil fertility (Gelfand & Robertson, 2015), weather (George et al., 1988), soil management (Oberson et al., 2007), presence of effective indigenous rhizobia (Weber et al., 1989; Hungria, 2015) and their interactions (George et al., 1988; Santachiara et al., 2017). For example, Purcell et al. (2004) found great sensitivity of the BNF process with drought; Schipanski et al. (2010) reported high soil inorganic N levels and/or excessive soil moisture to decrease N fixation, while Lindemann and Ham (1979) reported that soil temperatures in the range of 20 to 25 ^{o}C are optimal for nodule growth and thus BNF. These environmental factors are dynamic and difficult to control or predict in rainfed systems. In addition, different soybean genotypes differ in their BNF capacity (Patterson & LaRue, 1983; Herridge et al., 1990; Mastrodomenico & Purcell, 2012) creating a complex situation that makes BNF prediction challenging.

As soybean biomass production and yield increases, the gap between total N accumulation and BNF increases (Ciampitti & Salvagiotti 2018). Even when BNF is high (>300 kg N ha^{-1}), high-yielding soybean production fields may require additional N (Ciampitti & Salvagiotti 2018). The addition of N fertilizer could maintain high crop growth rates during reproductive stages, which is when the plant rapidly mobilizes N from leaves to seeds (Sinclair & de Wit, 1976; Wesley et al., 1998). However, with few exceptions (Rotundo et al., 2014; Cafaro La Menza et al., 2017), the majority of research indicates little-to-no benefit of N fertilization on yield (see Mourtzinis et al., 2018 for a synthesis of 207 soybean N-trials in the US).

In addition to crop yields, N balances in soybean production affect soil C balances and long term soil health and sustainability. If soybean is a net user of soil inorganic N (soil inorganic N uptake > BNF), both soil organic C and N stocks will decline (Christianson et al. 2012). Salvagiotti et al. (2008) found that 80% of the datasets collected from 1955–2006 averaged a net-negative N balance of –40 kg N ha^{-1}, when only aboveground N was only taken into account.). Long-term measurements of soil organic C and N stocks in rotated maize-soybean systems confirming a decline in soil C that would be expected if N outputs from soybean seed harvest exceed N inputs from BNF (Poffenbarger et al., 2017).

Biological N fixation is a key to both yield advances and long-term soil sustainability. Our knowledge of BNF relies almost exclusively on estimates of BNF taken at physiological maturity. These end-of-season measurements offer valuable information about N balances. However, they do not provide information about in-season N dynamics, which may help researchers optimize the timing of N fertilizer inputs when and if required by the crop for the benefit of the production and environmental quality (Liu et al., 2011). Few studies have measured BNF throughout the growing season (see Table S1 for an extensive review). In the USA, the most recent studies on BNF over the growing season were conducted in irrigated production regions such as Kansas, Nebraska and Arkansas (Salvagiotti et al., 2009; Mastrodomenico & Purcell, 2012, Cafaro La Menza et al., 2017; Tamagno and Ciampitti, 2017). In rainfed production regions of USA Midwest – which accounts for the 78% of USA soybean production and 37% of the global soybean production (USDA-NASS, 2017) – the few studies available are from 1980's or earlier, when cultivars and management practices were different than current pactices (e.g. narrower row spacings, higher poppulations and lower to No-N fertilizer application to soybeans; Allos and Bartholomew, 1955; Weber, 1966; Taylor, 1980; Berg et al., 1988; de Bruin and Pederson, 2008; USDA-NASS, 2019). The USA Midwest region and Iowa in particluar has deep, fertile soils (soil organic matter of 3–7%) and shallow water tables (Risso et al., 2018), making it quite different from the irrigated regions.

Given the importance of BNF to future yield gains and long-term soil sustainability as well as the limited data available from Iowa, we measured soybean BNF during the growing season along with the other soil and plant variables such as soil water and nitrate and biomass accumulation to:

1. Quantify the amount and fraction of BNF across locations, years and management treatments in Iowa
2. Determine when BNF is maximized during the season and its maximum value
3. Explore environmental factors and plant traits that explain variation in BNF

We hypothesized that BNF in Iowa soils will be less than the 55% mean value (n= 733 data points obtained in BNF studies from 1955-2016; Ciampitti & Salvagiotti, 2018) due to inherently high soil fertility and soil organic N mineralization, which is known to suppress BNF (Schipanski et al., 2010; Gelfand & Robertson, 2014). We also hypothesized that BNF will be maximized during the seed filling period because of the greater demand for N by soybeans during this time (Purcell et al., 2004). Lastly, we hypothesized that plant biomass will be the best predictor of BNF among other plant and envrionmental variables (Sinclair et al., 1987; Peoples et al., 2009).

2 Materials and Methods

2.1 Field experiments

We conducted two field experiments. In the first experiment, we measured soybean BNF and soil-plant variables at multiple times throughout soybean growth and development using the ^{15}N isotope dilution method (hereafter referred as the 'isotope dilution experiment') across two planting dates, two locations and two years, equal to eight datasets (Table 1). Different planting dates created different environments (i.e., each planting date had different weather).

In the second experiment, we measured soybean BNF at one-time (physiological maturity), using the ^{15}N natural abundance method (hereafter referred as the 'isoline experiment'). In this experiment we used N fixing (i.e., nodulating) and non-fixing (i.e., non-nodulating) soybean isolines, more details on Table 1. This experiment was used to complement results from the isotope dilution experiment (Unkovich et al., 2008). However, available isolines nodulating and non-nodulating isolines are not modern cultivars whereas for the isotope dilution method we used elite germplasm but requires assumptions about the type of soil inorganic N taken up by the soybean (i.e., NH_4, NO_3 or both). Chalk et al. (1996) and Unkovich et al. (2008) provide a detailed discussion about these methods

The isotope dilution experiment was carried out in 2015 and 2016 in central (42°01'14.9'' N, 93°46'31.2'' W) and northwest Iowa, USA (42° 55' 35.0" N 95° 32' 23.20"W). The USDA soil series (USDA NRCS, 2018) were Nicollet loam (fine-loamy, mixed, superactive, mesic Aquic Hapludolls) at the central site and Primghar loam (fine-silty, mixed, superactive, mesic Typic Endoaquolls) at the northwest site. Both soils had similar organic matter: 3.8% at the 0–30 cm, 2.5% at the 30–60 cm, and 1.3% at 60–90 cm depth (measured November 2014). According to the USDA soil survey, the available water capacity from 0 to 150 cm depth is 188 and 161 mm for the northwest and central sites, respectively, and both soils are characterized as poorly drained. Both sites had shallow water tables over the growing seasons that fluctuated from 50 to 200 cm (Ordonez et al., 2018a). The isoline experiment was carried conducted nearby (<500 m) the isotope dilution experiment in central Iowa site in 2016.

The central and northwest sites have an average summer temperature of 22.5 °C and 21.6 °C and cumulative precipitation during summer time of 330 mm and 355 mm, respectively (1986–2016; Iowa Environmental Mesonet 2017). The 2015 summer was wet in central Iowa (193 mm more rain than normal) and cold in northwest Iowa (2 ºC colder than normal), while the 2016 summer was warm (1.5 ºC warmer than normal) in central Iowa and dry (100 mm less rain than normal) in northwest Iowa (Fig S1).

Our experiments were managed for optimum soil fertility following Iowa State University recommendations (Mallarino et al., 2013), and soybean plots did not receive N fertilizer nor irrigation. We applied pesticides and herbicides as needed to control weeds, pests, and diseases. Soybean followed maize crops every year. In the isotope dilution experiment, soybean was planted in a completely randomized plot design with two planting dates, each replicated three times (Table 1). Each replicate was 278 m^2, and ^{15}N isotope was applied to unconfined microplots (3 rows x 1.33 m length; 3.45 m^2) situated within each plot. The application of ^{15}N was sufficient to alter the ratio of ^{15}N:^{14}N of the soil inorganic N pool so that it allowed us to use a two-pool mixing model to determine the proportion of BNF, but too little to have a fertilization effect (see Unkovich et al., 2008, section 2.2). In this experiment, we used commercial varieties with maturity groups 2.7 and 2.2 for the central and northwest site, respectively (Table 1). The isolines experiment was a two-way factorial complete-block randomized design including N fertilizer addition rate and timing (Table 1). In this experiment, we used soybean isolines nodulating and non-nodulating from Harosoy (maturity group 2.0), and M129 (maturity group 1.4).

2.2 Main experiment to determine BNF using the isotope dilution method

The ^{15}N isotope dilution method allows collection of time-integrated measurements of BNF in the field, providing estimates of the fraction of the total N uptake derived from BNF independent of the crop yield (Chalk & Ladha, 1999). ‘Dilution’ refers to the decrease of the soil inorganic ^{15}N label over time by the production of inorganic N from soil organic matter mineralization that is dominated by ^{14}N (Barraclough, 1991). The major assumption of this method is that the atom%^{15}N measurement of inorganic N in soil solution reflects the atom% ^{15}N of the soil inorganic N pool that the plant accesses, replacing the use of the non-fixing reference plant (Chalk et al., 1996). A more detailed description of this method can be found in Unkovich et al. (2008).

In each study year, we applied 99 atom% enrichment $^{15}NH_4{}^{15}NO_3$ isotope tracer at 8.70 kg N ha^{-1}, one month before planting. This allowed us to avoid the most rapid period of isotope dilution, providing a more stable ^{15}N signal in the soil inorganic N pool over time. Application of the label followed Sanchez et al. (1987). We used a backpack hand sprayer with a compressed CO_2 tank set at a pressure of 60 psi. The labeled isotope tracer was mixed with green dye to help us visualize the applied area and ensure a homogeneous application.

2.2.1 Crop measurements

In each plot during the growing season, we measured: plant development, density, aboveground biomass production and partitioning, leaf area, N concentration and atom% ^{15}N of the aboveground plant organs. At the end of the growing season, we used a combine harvester to measure final yields (adjusted to 130 g kg^{-1} moisture).

Within each microplot we measured BNF throughout the growing season; seven times in 2015 (V3 to R6.5 growth stage, i.e., 3rd trifoliate leaf to beginning of physiological maturity; Fehr et al., 1971) and ten times 2016 (V3 to R8 growth stage). At each sampling time, we collected three whole plants (middle row, shoot plus root biomass), leaving five plants in between each sampling to avoid border effects. .Microplot samples were used to measure organ N concentrations and atom% ^{15}N. Also, we collected 1 m^2 plants from the plots to measure biomass production on an area basis and the partitioning among organs.

Root biomass was also collected at the same dates as aboveground biomass in the microplots in both years by digging an area of 25 x 25 cm with a spade (Gelfand & Robertson, 2014). In 2015, the root sampling depth was 30 cm from V3 to the R3 stage and increased over time to 80 cm depth at the R6.5 stage. In 2016 root sampling depth was guided by Ordóñez et al. (2018a, b) and ranged from 40 cm at the V3 growth stage to 80 cm at and after the R4 growth stage. Supplementary figure S5 shows root depths for the year 2016. Root depth measurements were taken 1-2 days before BNF measurements.

In the laboratory, soybean plants from entire plots and microplots were partitioned into seeds, pod shells, leaves, stems and petioles, and roots (including nodules) although not all organs were present at all the sampled growth stages. Soybean organs were oven dried at 60 °C to constant mass, weighed, and ground for C and N analyses using dry combustion elemental analysis (LECO C and N analyzer; LECO Corporation, St. Joseph, Michigan). The N isotope ratio of individual soybean organs was determined with isotope ratio mass spectrometry.

2.2.2 Soil measurements

Decagon sensors were used to measure hourly volumetric soil water content and temperature at 15 cm depth (for a description see Togliatti et al., 2017). Soil samples were collected on the same day as plant samples. In each ^{15}N-labeled microplot, a composite sample of 8 soil cores were collected to measure soil inorganic N concentrations and atom% ^{15}N. Within each plot a composite sample of 12 soil cores were sampled for soil NO_3-N and NH_4-N determinations. Both samples were made with a 2 cm diameter soil core. Soil samples in the microplots were collected to the same depth as the roots samples so that our atom% ^{15}N measurement of the soil inorganic N pool represented the N pool that the plants accessed; as a result, the depth of samples changed during the course of soybean growth. All soil samples were stored and transported in coolers kept at a 4 °C and processed immediately .

Ammonium (NH_4^+) and nitrate (NO_3^-) concentrations of field moist soil samples (plots and microplots) were extracted with reciprocal shaking in 2 M KCl (5:1 solution/soil ratio) and followed the protocol of Hood-Nowotny et al. (2010). The atom% ^{15}N of the NH_4+NO_3 in 2 M KCl soil extracts from the microplots was determined by isotope ratio mass spectrometry after diffusion to filter paper using reagents, blanks, and check-standards according to Stark and Hart (1996). In this procedure, both the NH_4^+ and NO_3^- were diffused and analyzed simultaneously which assumes that plants access NH_4^+ and NO_3^- in proportion to their relative abundances in the soil. Our methods reflect the consensus that annual crops use little organic N, moreover, the discrimination between N isotopes during the process of nitrification is extremely small relative to the difference in ^{15}N enrichment between the soil inorganic N pool and the atmosphere ^{15}N pool (Högberg, 1997) and, in N rich agricultural soils, most NH_4^+ is transformed to NO_3^- (Booth et al., 2005).

2.2.3 BNF calculations

The atom% ^{15}N of total aboveground biomass for each measurement was calculated as a weighted mean based on the proportion of total plant N in each organ. The atom% ^{15}N of soil inorganic N was normalized to a 30 cm depth at which most of the soybean roots were concentrated according with Ordonez et al. (2018a); and then smoothed by fitting a 3-parameter exponential decay function over time (Fig. S2) similarly as Chalk et al. (1996). Fitted soil atom% ^{15}N were integrated linearly using the corresponding proportion of total plant aboveground N accumulation at each sampling time (i.e., stage; Fig.1), done for eachplot. Using these data, the amount of BNF was estimated by using the isotope two-pool mixing model for each stageusing the ^{15}N isotope dilution method , similar as Unkovich et al. (2008):

$$\mathrm{BNF_{IDM}} = \left[\frac{(\text{atom\% }^{15}\text{N soybean} - \text{ atom\% }^{15}\text{N air})}{(\text{atom\% }^{15}\text{N soil inorganic} - \text{ atom\% }^{15}\text{N air}}\right] * \mathrm{TN} \qquad (1)$$

where $\mathrm{BNF_{IDM}}$ corresponds to the amount of BNF (kg N fixed ha^{-1}) determined by the ^{15}N isotope dilution method in each sampling. The atom% ^{15}N of soybean corresponds to the integrated soybean aboveground biomass ^{15}N; atom% ^{15}N air is equal to 0.3663; atom% ^{15}N soil corresponds to the soil ^{15}N enrichment, and TN is the amount of N accumulated in the aboveground biomass measured on each corresponding sampling.

2.3 Complementary experiment of BNF measured using soybean isolines

For the second experiment we used the ^{15}N natural abundance method (Shearer and Kohl, 1986), including near-isolines soybeans (i.e., nodulating and non-nodulating) assumed to have similar plant growth and development. Soybean isolines nodulating are BNF capable, whereas non-nodulating only to takeup N from the soil. Soybean BNF was only measured at the beginning of physiological maturity (i.e., R6.5) of Harosoy and M29 isolines.

2.3.1 *Crop measurements*

The measurements collected: atom% ^{15}N of plant organs, root samples for nodule counts, biomass and partitioning into different organs, tissue C and N concentrations. Methods for these measurements were the same as in section 2.2.1. The harvest area was 1 m^2 per plot. In addition, we counted the presence of nodules in nodulating and non-nodulating soybean isolines and discarded non-nodulating plants that produced nodules.

2.3.2 *BNF calculations*

The estimation of the BNF amount in the second experiment was done only at the R6.5 stage by input the measured atom% ^{15}N isotope ratios of non-nodulating and nodulating soybean isolines in a two-pool mixing model and multiplied this fraction by the total aboveground N accumulated in the nodulating plant at R6.5 stage similar as (Unkovich et al., 2008):

$$\mathrm{BNF_{INA}} = \left[\frac{(\mathrm{atom\%}\ ^{15}\mathrm{N\ of\ nonNod}) - (\mathrm{atom\%}\ ^{15}\mathrm{N\ of\ Nod})}{(\mathrm{atom\%}\ ^{15}\mathrm{N\ of\ nonNod}) - \mathrm{B}}\right] * \mathrm{TN} \qquad (2)$$

where BNF_{INA} corresponds to the total amount of BNF (kg N fixed ha^{-1}). The atom% ^{15}N of non-Nod and Nod are the weighted aboveground biomass atom% ^{15}N from non-nodulating and nodulating soybean plants, respectively, calculated by multiplying the atom% ^{15}N of each plant organ with its corresponding proportion of total aboveground N accumulation, for each isoline within each plot. The B-values were 0.3655 atom% ^{15}N (-2.26 $\delta^{15}N$) for M129 isoline (Schipanski et al., 2008), and 0.3656 atom% ^{15}N (-1.97 $\delta^{15}N$) for the Harosoy isoline (Balboa & Ciampitti, unpublished data, 2018), both reported from aboveground biomass collected at R6.5 stage in N fixing soybean plants. We used the aforementioned B-value to correct for the within-plant fractionation of ^{14}N and ^{15}N between aboveground and nodulated roots (Unkovich et al., 2008).

2.3 Calculations and statistics

We calculated soybean N content for each plant organ by multiplying tissue dry matter by its corresponding N concentration. Soybean protein concentration was calculated by multiplying seed N concentration by 6.25 and expressed as a percentage. Time series aboveground biomass production, N accumulation and cumulative BNF data were fitted to a 3-parameter logistic equation (Archontoulis & Miguez, 2015) using R software (R Core Team, 2018), which allowed us to smooth all data points. Then, using the derivatives, we calculated the corresponding daily rates for each variable throughout the growing season and for each environment. The non-linear model fitted to the data had $R^2 > 0.96$ in all cases (Fig. 1).

$$Y = \frac{Ymax}{1 + e^{[-k\,(t - tm)]}} \tag{3}$$

where Y corresponds to the response variable either aboveground biomass production, N accumulation or BNF data (reported in kg ha^{-1}). The coefficients t corresponds to the day of the year (DOY), Y_{max} is the asymptotic or maximum Y value, tm is the inflection point at each growth, aboveground N accumulation and BNF rate is maximized. And, k controls the steepness of the curve (Archontoulis & Miguez, 2015). All parameter values and metrics of goodness of fit are provided in the supplementary materials, Table S2.

At physiological maturity we calculated N accumulation efficiency (or nitrogen used efficiency) as the ratio between N accumulation and biomass and N fixation efficiency as the ratio between N fixation and biomass. A partial N balance was calculated as the difference between the aboveground BNF minus N harvested in soybean seeds, reported in kg N ha^{-1} (Salvagiotti et al., 2008). The difference between total N accumulation and N fixation rate equals the amount of N derived from the soil. Plant N remobilization rate was calculated as N accumulation rate in seeds and pods minus soil N uptake and fixation rate. Soil inorganic N uptake rate equal to total aboveground N accumulation rate minus BNF rate. To examine relationships between BNF and crop/environmental variables we used regression (PROC REG) in SAS version 9.4 (SAS Institute, Cary, NC, USA).

Differences in yields, BNF, aboveground N accumulation, %BNF, seed protein, among treatments in the isotope experiment, were deemed significant at α= 0.05. We used PROC GLIMMIX and the lsmeans statement in SAS which makes a pairwise comparison among the treatment means. The isoline experiment was analyzed as a pseudo factorial experimental design in which we compared all the interactions (isolines x N-rate x N-timing; Table 1) and referred to them as treatments. For statistical analyses, we only used the nodulating isolines of Harosoy and M129 by using PROC GLM in SAS. Additionally, we used contrasts to define differences between isolines, N-rate, and N-timing treatments. Differences were deemed statistically significant at the α= 0.05 level.

3 Results

3.1 Environmental characterization and treatment differences

Cumulative precipitation from planting to beginning of physiological maturity across the eight environments (2 years x 2 sites x 2 planting dates) varied from 335 to 626 mm (coefficient of variation, CV= 25%; Fig. 2b). The cross-environment variation in soil moisture at 0-15 cm depth (CV = 9.5%) was less than precipitation and the average growing season topsoil moisture ranged from 0.22 to 0.30 mm mm^{-1}, which is near field capacity. Topsoil temperature at 0-15cm depth ranged from 19 to 23°C (Fig. 2a) closely following air temperature. At planting, the average top soil inorganic N ranged from 34 to 130 kg ha^{-1}; the highest values were recorded in the year 2015 at the northwest location (both early and late plantings) because the maize crop in the previous year was over-fertilized (Fig. 2c).

Seed yield, total N accumulation, and protein concentrations were significantly different across the eight environments ($p<0.001$; Table 1). In the isotope dilution experiment, the average yield was 4.3 Mg ha^{-1}, total aboveground BNF was 131 kg N ha^{-1}, aboveground N accumulation was 298 kg N ha^{-1}, and seed protein concentration was 32% (Table 1). In the isoline experiment, yield, BNF, and aboveground N accumulation mean values were lower than the isotope dilution experiment (Table 1). The N fertilizer effect on the above variables was not statistically significant (Table 1).

3.2 Soybean BNF rates and timing of maximum rate

Crop growth, aboveground N accumulation, and aboveground BNF rates reached maxima at different times during the growing season and varied across the eight datasets in the isotope dilution experiment (Fig. 3). Crop growth rates peaked around the R4 stage (DOY 214 ± 3; July 27 to August 9), followed by BNF rates at R5 stage (DOY 222 ± 4; July 28 to August 24) and N accumulation rate at R5±0.5 stage (DOY 232 ± 4; August 10 to September 4; Fig. 3). Seed and pod-wall dry matter accumulation rates were maximized at the R6 stage (data not shown). Maximum crop growth rates varied from 138 to 213 kg dry matter ha^{-1} day^{-1}, BNF rates from 1 to 3 kg ha $^{-1}$ day^{-1}, and N accumulation rates from 4 to 6 kg ha^{-1} day^{-1} (Fig. 3). Among these processes, N fixation rate was the most variable (3-fold increase in variation) followed by crop growth and N accumulation.

Soybean biologically fixed 2-times more N during the reproductive stages (R2.5 to R7; average of 89 kg N fixed ha^{-1}, 68% N fixed) than during the vegetative stages (Fig. 4; average of 42 kg N ha^{-1}, 32% N fixed). Across the two years, the amount of BNF in vegetative stages was similar, but the amount of BNF in reproductive stages was different. For instance, BNF in 2016 during the pod-seed filling period was 1.6-times greater than in 2015 (110 vs. 71 kg N fixed ha^{-1}, respectively; Fig. 4). Additionally, measurements collected in 2016 from planting to harvest, showed that BNF beyond R6.5 was small, representing 6% of the total N accumulation in 2016.

3.3 Correlation between BNF, plant traits and environmental factors

There were strong linear relationships between in-season biomass accumulation, N accumulation, and BNF ($R^2 = 0.83$ to 0.94; $p< 0.01$; Fig. 5a). The linear nature of the relationship indicates that both N accumulation and BNF are proportional to biomass accumulation at a constant rate. The slope (a measure of efficiency) was 0.035 kg N accumulation per kg biomass and 0.013 kg N fixed per kg biomass (Fig. 5a). The efficiency of BNF and N accumulation were similarly variable (CV = 21%) across the eight datasets. Significant correlations were also found by considering only measurements obtained at physiological maturity from both experiments (i.e., isotope dilution and isoline experiment; Fig. 5b). Compared to the in-season data, the BNF efficiency was similar, but the N accumulation efficiency was lower; both as a function of aboveground biomass (Fig. 5c). The difference in N accumulation efficiency was caused by variation in tissue N accumulation at maturity and time of sampling as evidenced by the multiple N accumulation data points around the stage of maximum biomass production (Fig. 5a).

Both in-season and end-of-season data indicated that the gap between total N accumulation and BNF increases with increasing biomass production (Fig. 5a and b; biomass > 4,000 kg ha^{-1}). The gap becomes quite variable after the R5 stage (Fig. 6 and S4a). To understand the causes of this variability we explored two potential drivers: C supply from photosynthesis (Fig. 6b) and N demand from reproductive organs (Fig. 6c). We found that BNF is related to C supply but not to N demand. The minimum daily crop growth rate required to sustain a maximum BNF rate was around 135 kg dry matter ha^{-1} day^{-1} (Fig. 6b). At lower crop growth rates, BNF decreased. The opposite results were found for the soil inorganic N uptake rate that was related to reproductive organ N demand but not to C supply (Fig. 6c). We also found that when the reproductive organ N demand was not satisfied by BNF and soil inorganic N uptake, N remobilization from vegetative to reproductive plant tissues took place following an exponential pattern (Fig. 6c; $R^2 = 0.74$). Thus, as the crop progresses in reproductive stages and the rate of biomass production decreases (Fig. 3), the contribution of BNF to the total N accumulation declines (Fig. 6b). Given that soil inorganic N uptake was not dependent on crop growth rate during seed fill period (Fig. 6b), this created the larger N gap between BNF and total aboveground N accumulation towards maturity (Figs. 5 and 6a). Moreover, an increase in soil N mineralization late in the season due to rainfalls may also contributed to the decline in BNF during late reproductive stages.

Soybean end-of-season yield data (Table 1) combined with literature observations (Table S1) showed a significant relationship with BNF (Fig. 7a) but with a lower R^2 compared to biomass. Similarly, the relationship between cumulative BNF and total aboveground N accumulation was significant (Fig. 7b). On the other hand, harvest index, partial N balance, N accumulation efficiency and seed protein concentration were not significantly related to BNF (Fig. 7). Regarding environmental factors, regression analysis between BNF and average soil temperature, moisture, N, and radiation during the growing season indicated no significant relationships in the isotope dilution experiment (Fig. S3). Thus, among several factors explored in this study, the plant factors, and in particular biomass, were the best predictors of BNF.

4 Discussion

4.1 Comparison between our BNF measurements and literature data

Across the eight environments, BNF accounted for 45% (sd ± 13%) of the total aboveground N accumulation, which is below the mean value of 55% (sd ± 21%) reported by Ciampitti and Salvagiotti (2018). These results are not surprising given that Iowa soils have high rates of N mineralization (Osterholz et al. 2017) and high levels of inorganic N are known to suppress BNF (Schipanski et al., 2010). However, the variability around the observed BNF average value was high (range: 73 to 176 kg N fixed ha^{-1} day^{-1}), which demonstrates that use of an average value for BNF can lead to unreliable N budget calculations. Typically, N budget calculations assume the amount of N harvested in the seed is equal to the amount of BNF (IPNI, 2012). We believe the use of biomass data to estimate BNF could improve accuracy in N-budget calculations (see regressions in Figs. 5).

Based on previous estimates of soybean BNF in Iowa, our results demonstrate that the proportion of BNF to total aboveground N accumulation has not changed, but the total amount of BNF has increased by almost 100% (Webber, 1966; Berg et al. 1988). The differences could be attributed to the higher yield levels, which also integrates genotypic, environmental and management differences (cultivars, planting density, etc.; Balboa et al., 2017). Moreover, soybean protein concentrations in our study were in general low, but fall within the range of the latest report for soybean in USA Midwest reported by Tamagno et al. (2018) and Assefa et al. (2019).

4.2 Time when BNF is maximized during the season and the maximum value

In this study, BNF was maximized early in the seed filling period and then decreased probably by a lower daily C supply from photosynthesis despite the high N demand imposed by seed N accumulation during the middle-late seed fill period (Figs. 3b and 6). In the literature, the timing of BNF rate maximization is not consistent. Some studies report maximum rates during seed fill (Deibert et al., 1979; Zapata et al., 1987; Mastrodomenico & Purcell, 2012), while others report maximum rates around flowering (Lawn & Brun, 1974; Thibodeau & Jaworski, 1975; George & Singleton, 1992). The reason for the inconsistency could be related to genotype, management, environmental differences among the aforementioned studies or even methodological issues such as method used to estimate BNF or method used to derive rates (e.g. use of primary data or use of a nonlinear equation). It should be noted that the period around flowering coincides with the period of maximum nodule activity (Guafa et al., 1993; Gan et al., 1997; 2002), while the seed filling period coincides with the time of highest N accumulation by soybean plants (Hanway & Weber, 1971; Harper & Gibson, 1984; Purcell et al., 2004).

Our estimates for the maximum BNF rate fell within the lower range suggested by Unkovich and Pate (2000) and other investigators (3.8 kg N fixed ha^{-1} d^{-1}; Zapata et al., 1987; Mastrodomenico & Purcell, 2012). Our estimate was half that compared to a rate of 5 kg N fixed ha^{-1} d^{-1} measured in a sandy and low organic matter soil in Florida (DeVries et al., 1989), but similar to 2.7 kg N fixed ha^{-1} d^{-1} rate estimated in irrigated fields in Nebraska by Salvagiotti et al. (2009). We atribute this difference to the high soil organic matter levels of our experimental sites. Chen et al. (2016) reported that crop models are very sensitive to the potential N fixation rate.

The maximum N accumulation rate was reached soon after the maximum BNF rate (Fig. 3b). We attributed this to the contribution of soil inorganic N during the seed fill period (Fig. 6c). We estimated a maximum soil inorganic N uptake rate of 4.6 kg N ha^{-1} day^{-1}, which is comparable to estimates from a previous study of maize in this region (Osterholz et al. 2017). In our experiments, air and soil temperatures were similar between the vegetative and reproductive periods, but precipitation was somewhat higher during the reproductive stages thus favoring soil organic matter mineralization (Fig. 2b). During seed fill, most of the high C-to-N ratio maize residue from the previous crop that immobilizes N during decomposition is smaller compared to early vegetative stages. This could mean that more N from soil organic matter mineralization was available for root N uptake during seed fill period as shown in Fig. 6. During that period the plant demand for N was high, which stimulated high soil N uptake rates (Fig. 6c).

Before seed fill, both BNF and soil inorganic N uptake were related to crop growth rate (R^2 >

0.58; data not shown), however, during seed fillBNF was more sensitive to crop growth rate than the soil inorganic N uptake process (Fig. 6). Previous studies have focused on N fertilization additions as a way to increase N accumulation and thus yields but without success (Mourtzinis et al., 2018). Nitrogen fertilization alone cannot maintain high crop growth rates if other factors such as soil moisture are limiting photosynthesis. Results from this study suggest that more focus should be placed on increasing C availability (green and healthy canopy and soil moisture near field capacity) rather than just soil inorganic N availability towards increasing fixation. Increasing C will require a systems approach to concurrently evaluate and optimize genotype x management x environment interactions. For example, Boote et al. (2003) demonstrated the impact of different plant traits (e.g. specific leaf weight, root front velocity) on soybean yields under different management scenario. An increase of BNF will have positive effects on both yield and N budgets, thus on environmental sustainability (Fig. 6).

4.3 Environmental and plant factors explain BNF variability

We found that soybean biomass was the best predictor of BNF compared to the many other explanatory variables that we tested (Figs. 7 and S3). The close link between BNF and biomass has been observed in previous studies for many legume species (Peoples et al., 2001, 2009; Soltani et al., 2006; Salvagiotti et al., 2008; Unkovich et al., 2008). The reason for the strong relationship between BNF and biomass is complex. BNF can increase leaf nitrogen and thus leaf photosynthesis (up to a point) and thus biomass production. On the other hand, a high photosynthesis can increase C supply and thus enhance BNF. The feedback between BNF and photosynthesis is detailed in Kaschuk et al. (2010). In a field study, De Bruin et al. (2010) found that soybean yields in Iowa increased with increasing photosynthetic rate during grain fill period. Another reason for the strong coupling between BNF and biomass is that some of the factors affecting BNF (e.g. soil moisture) also affect leaf photosynthesis and biomass production in the same direction (Peoples et al., 2009; Liu et al., 2011).

Analysis of environmental factors such as soil moisture–temperature–inorganic N and radiation effects on cumulative BNF were not significant, possibly due to few data points available (Fig. S3). Future studies need to explore more diverse environments in order to sufficiently study environmental response on soybean BN. Nevertheless, the observed trends agree with literature findings that there is a negative relationship between BNF and soil inorganic N (Purcell & Sinclair, 1990; Salvagiotti et al., 2008; Schipanski et al., 2010), and a positive relationship between BNF and increasing soil temperature or moisture (Lindemann & Ham, 1979; Purcell et al., 2004).

Of particular note is that our study region is characterized by shallow water tables that vary from 50 to 200 cm below the soil surface (Ordonez et al., 2018a). We believe this is one reason for top soil moisture (0–30 cm) being at 70 to 95% of field capacity over the entire growing season (Figs. 2 and S3). As mentioned earlier, precipitation was greater during the second half of the growing season, benefiting soil N mineralization. However, higher precipitation during that period probably caused water tables to rise and created oxygen deficit conditions. The impact of excessive moisture, and thus depletion of oxygen on the BNF process is not well understood (Bacanamwo & Purcell, 1999; Schipanski et al., 2010), therefore it deserves further research given also the expected increase in future spring precipitations in this region (Mellilo, 2014).

Data from this study, as well as data from 15 other studies showed a net negative partial N balance (seed N export greater than BNF; Tables 1 and S1). Only four studies resulted in positive partial N balance, all of which used the ureide method to quantify BNF (Table S1). In our calculations of partial N balance we excluded the contribution of roots. Even if we include the contribution of roots N (~31 ± 4 kg N ha^{-1}; Ordonez unpublished data from Iowa; n=12 environments during 2016–2018 in Iowa), the N balance is still negative. The inclusion of roots N offset the negative balance by 15%. Beyond our experiments, Ciampitti and Salvagotti (2018) reported an average negative aboveground N balance of 47 kg N ha^{-1}. For this balance to be neutral or positive the root N should be > 47 kg N/ha, which is unlikely based on our measurements and also literature data (Gelfand & Robertson, 2015)

The two approaches we used to measure BNF in soybeans are considered among the most accurate field methods (Unkovich et al., 2008). We consistently measured negative partial N balances in both experiments using different methods, confirming recent findings that N harvested in soybean seed exceeds BNF (Salvagiotti et al., 2008; Ciampitti & Salvagiotti, 2018).

5 Conclusion

The new in-season data and analyses presented here for deep, fertilize soils in the rainfed US Midwest soybean production region fill an important knowledge gap and have potential to assist, N budget calculations, and support crop growth model enhancement and testing. Major findings from this research include: i) soybean BNF contributed 45% (range: 23–65%) of the total aboveground N accumulation in Iowa, a region with deep fertilize soils and shallow water tables; ii) soybean N fixation can supply up to 3 kg N ha^{-1}d^{-1} while soil inorganic N can supply up to 4.6 kg N ha^{-1}d^{-1} in this region. The BNF rate was more sensitive to C supply from photosynthesis rather than N demand during the seed fill phase; iii) biomass accumulation was the best predictor of BNF among other variables tested such as soil inorganic N or moisture. Soybean partial N budget analysis, N in seeds minus BNF, indicated a negative balance even when we include the root N. Future research efforts should focus more on increasing C availability during the seed fill period rather than just soil inorganic N availability to produce greater soybean yields and maintain environmental sustainability.

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Tables

Table 1. Experimental details such as location, year, planting date, beginning of physiological maturity date (R6.5), stand count, cultivar name, yield, cumulative aboveground N fixed until beginning of physiological maturity, total aboveground N accumulation at R6.5, percent of total N derived from N fixation, and seed protein concentration for both isotope dilution and isoline experiments (nodulating and non-nodulating).

No.	Location (L)	Year (Y)	Planting date (PD)	R6.5 Date	Plants per m^2	N-Rate (kg/ha)	Timing-N	Cultivar name	Yield (Mg/ha)		BNF (kg N/ha)	N accum. (kg N/ha)		BNF (%)	Prot (%	
Isotope dilution experiment																
1	Central	2015	1-May	1-Sep	37	0	none	P92Y75	4.2cd		154	321c		48	33	
2	Central	2015	25-May	11-Sep	37	0	none	P92Y75	3.4e		109	311e		35	42	
3	Central	2016	6-May	25-Aug	30	0	none	P92Y75	4.4bc		156	241g		65	32	
4	Central	2016	3-Jun	15-Sep	42	0	none	P92Y75	3.6e		150	276f		54	30	
5	Northwest	2015	30-Apr	26-Aug	37	0	none	P22T61R	4.9a		115	327b		35	33	
6	Northwest	2015	25-May	10-Sep	36	0	none	P22T61R	4.1d		73	313d		23	34	
7	Northwest	2016	7-May	13-Sep	29	0	none	P22T61R	4.8a		113	241h		47	27	
8	Northwest	2016	1-Jun	13-Sep	32	0	none	P22T61R	4.6ab		176	353a		50	26	
								Avg.	4.30		131	298.00		45	32.	
								p-value (Y*L*PD)	<0.001		0.167	<0.001		0.124	0.0	
								CV (%)	12.95		37	13.70		29	17.	
Isolines experiment									*Nod.*	*Non-nod.*	*Nod.*	*Nod.*	*Non-nod.*	*Nod.*	*Nod.*	*N*
9	Central	2016	20-May	6-Sep	32	0	none	Harrosoy (nod)	3.0	0.9	127	168	44	76a	29	
10	Central	2016	20-May	6-Sep	36	0	none	M129 (nod)	3.1	1.7	108	172	97	63ab	30	
11	Central	2016	20-May	6-Sep	33	135	planting	Harrosoy (nod)	2.3	2.4	69	175	138	39c	33	
12	Central	2016	20-May	6-Sep	35	135	planting	M129 (nod)	3.4	2.1	135	222	158	61ab	30	
13	Central	2016	20-May	6-Sep	37	135	R1	Harrosoy (nod)	2.4	2.0	70	175	112	40c	30	
14	Central	2016	20-May	6-Sep	36	135	R1	M129 (nod)	2.1	1.2	68	153	86	45bc	33	
15	Central	2016	20-May	6-Sep	40	135	R4	Harrosoy (nod)	2.1	1.9	88	148	108	60ab	31	
16	Central	2016	20-May	6-Sep	38	135	R4	M129 (nod)	3.4	1.6	81	188	73	43c	30	
								Avg.	2.7		93	175		53	31	
								Factor / p-value								

Isoline (I)	0.088	0.574	0.516	0.899	0.52
N-time (N)	0.952	0.138	0.739	<0.001	0.685
R-rate (R)	0.991	0.505	0.699	0.282	0.373
(I*N*R)	0.819	0.315	0.95	0.001	0.878
CV (%)	21	29	13	25	5

(*) Yields reported at 130 g kg^{-1} moisture.

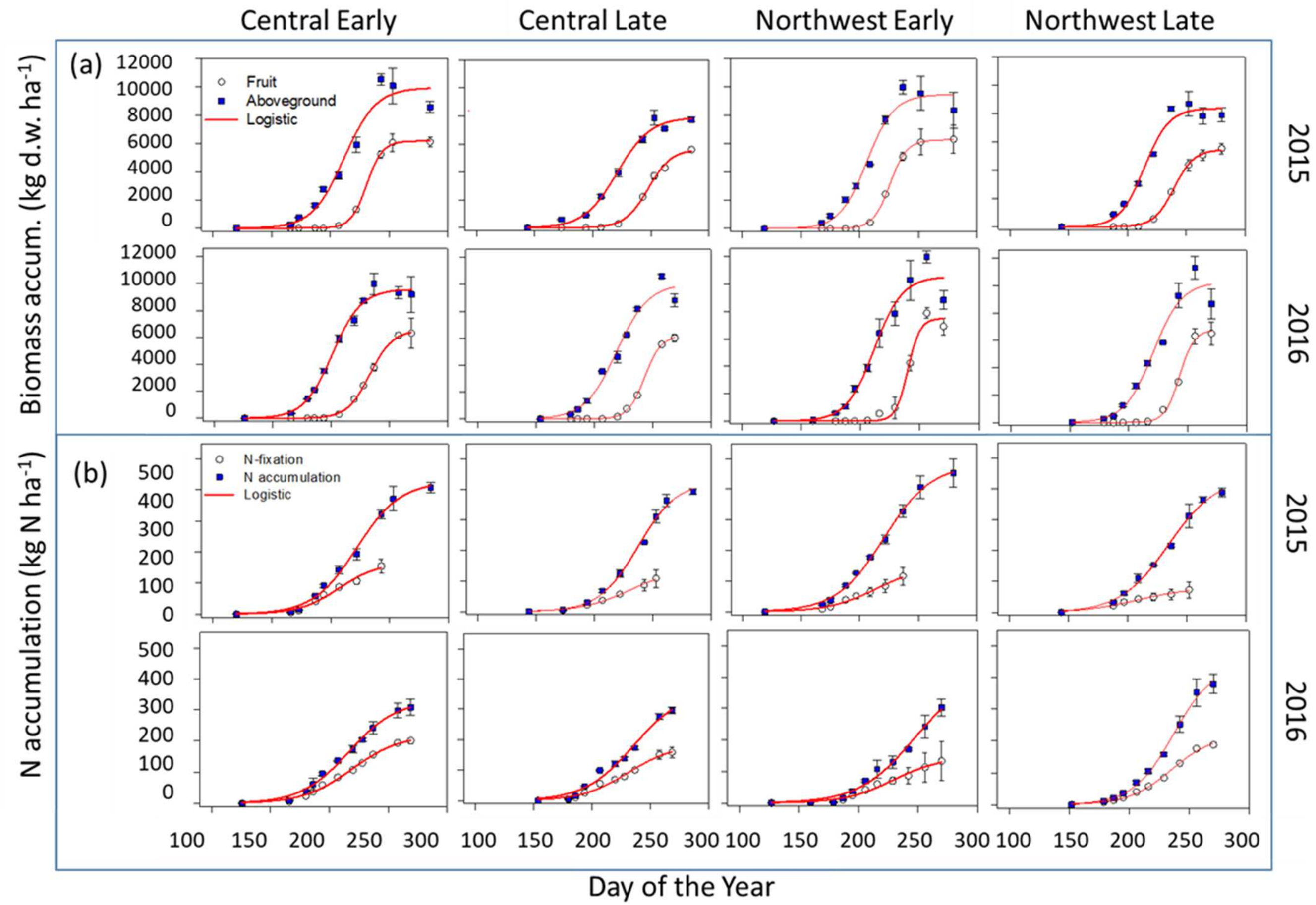

Figure 1. Cumulative soybean aboveground biomass matter (panels a), and aboveground N accumulation (panels b) throughout the growing season in all isotope dilution datasets. Each data point is the average of three replications. Red line represents the modified logistics function [$Y = Ymax/(1 + exp^{-k(t-tm)})$] fitted to the data. All parameters coefficients for each graph are located in Table S2.

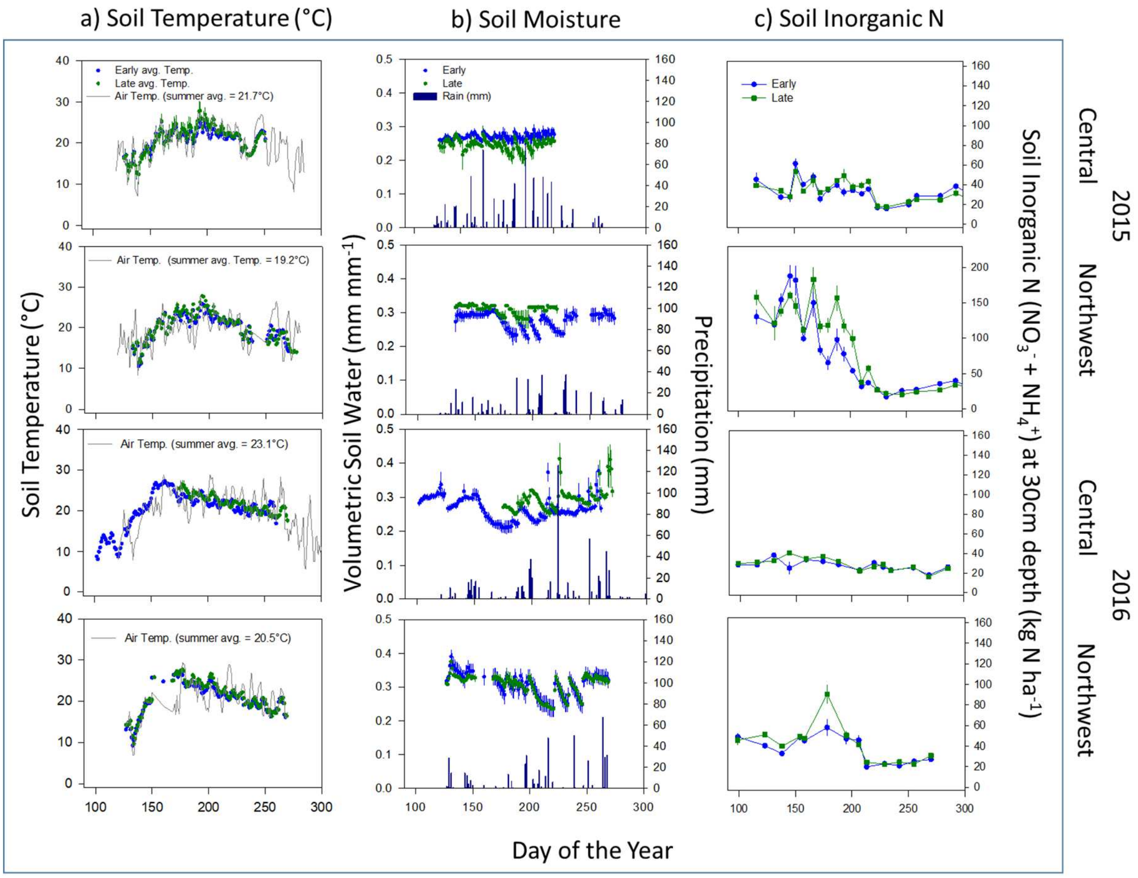

Figure 2. Weather and soil conditions at the experimental sites: soil temperature (a), and volumetric soil moisture at 0-15cm depth (left y-axis, b), precipitation (right y-axis, b), and soil inorganic N concentration at 0-30cm depth (c). Light grey solid line represents air temperature (a), blue and green dots represent measurements for early and late planting date treatments, blue bars are precipitation measurements throughout the growing season in both sites during 2015-2016.

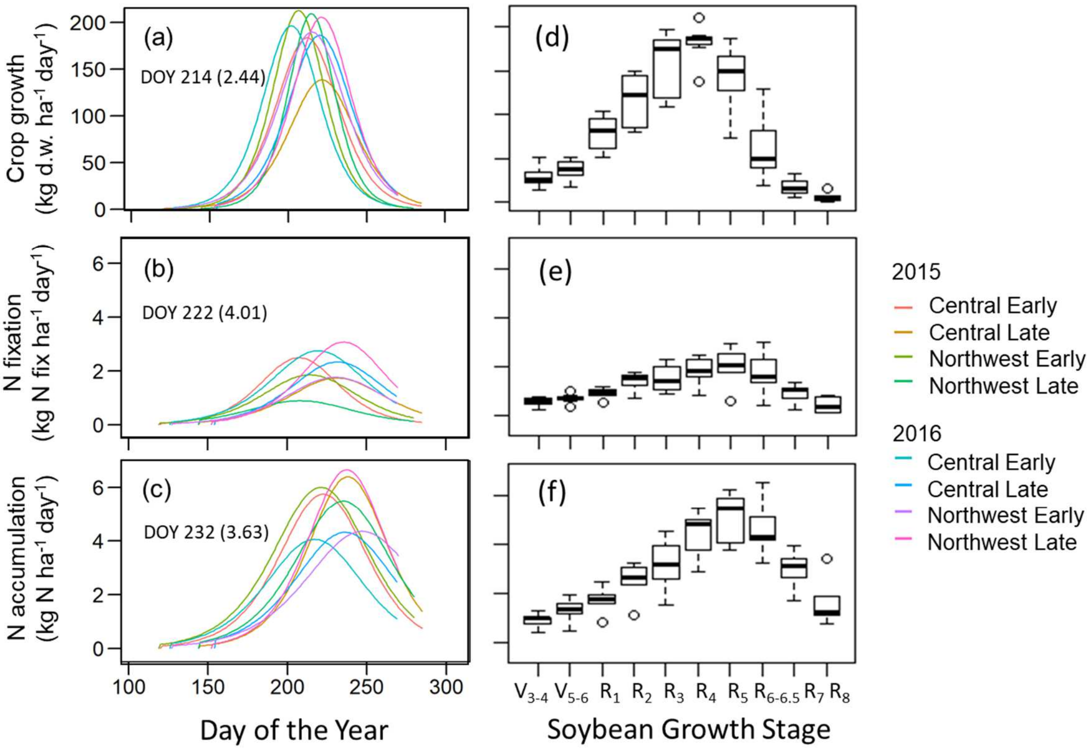

Figure 3. Left panels: estimated rates of crop growth (a), aboveground BNF (b), and aboveground N accumulation (c) as a function of day of year (DOY) from the isotope dilution experiment in all eight environments (2 sites x 2 planting dates x 2 years). Right panels: variation of actual rates of soybean processes as a function of different growth stages.

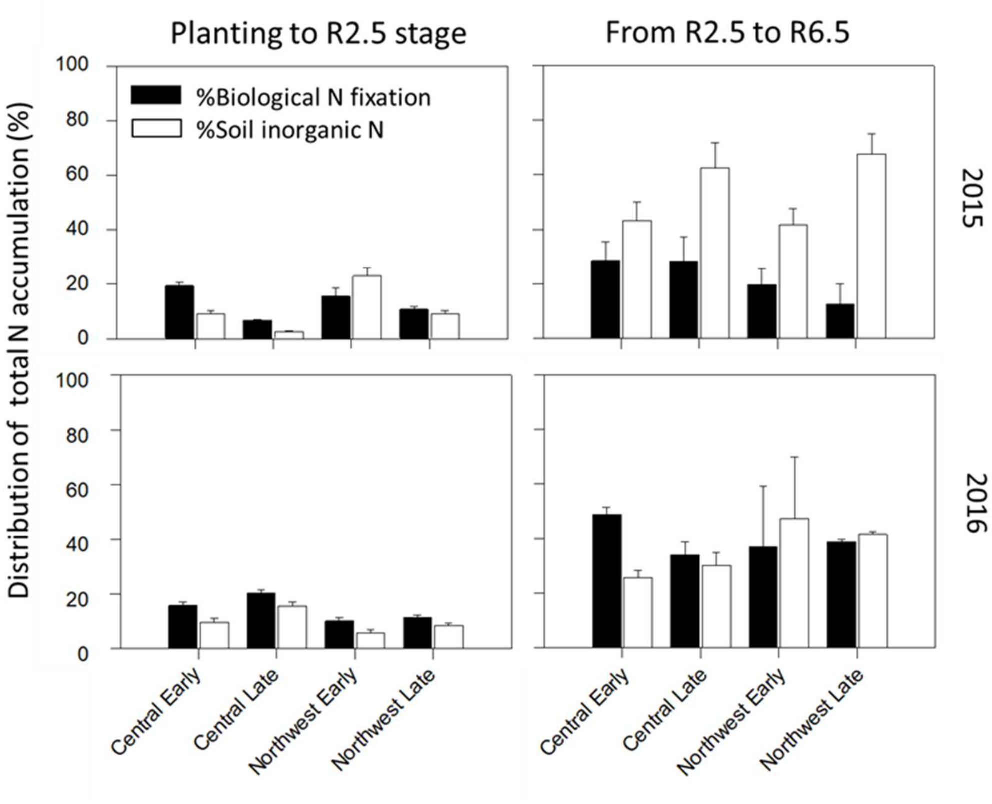

Figure 4. Distribution of soybean aboveground N accumulation derived from two sources (BNF and soil inorganic N) measured in central- and northwest-Iowa in early and late planting treatment during 2015-2016, respectively, from the isotope dilution experiment. Proportions reported from planting to R2.5 stage, and from R2.5 stage to R6.5 stage. Values are means of three replications per year, site and planting date.

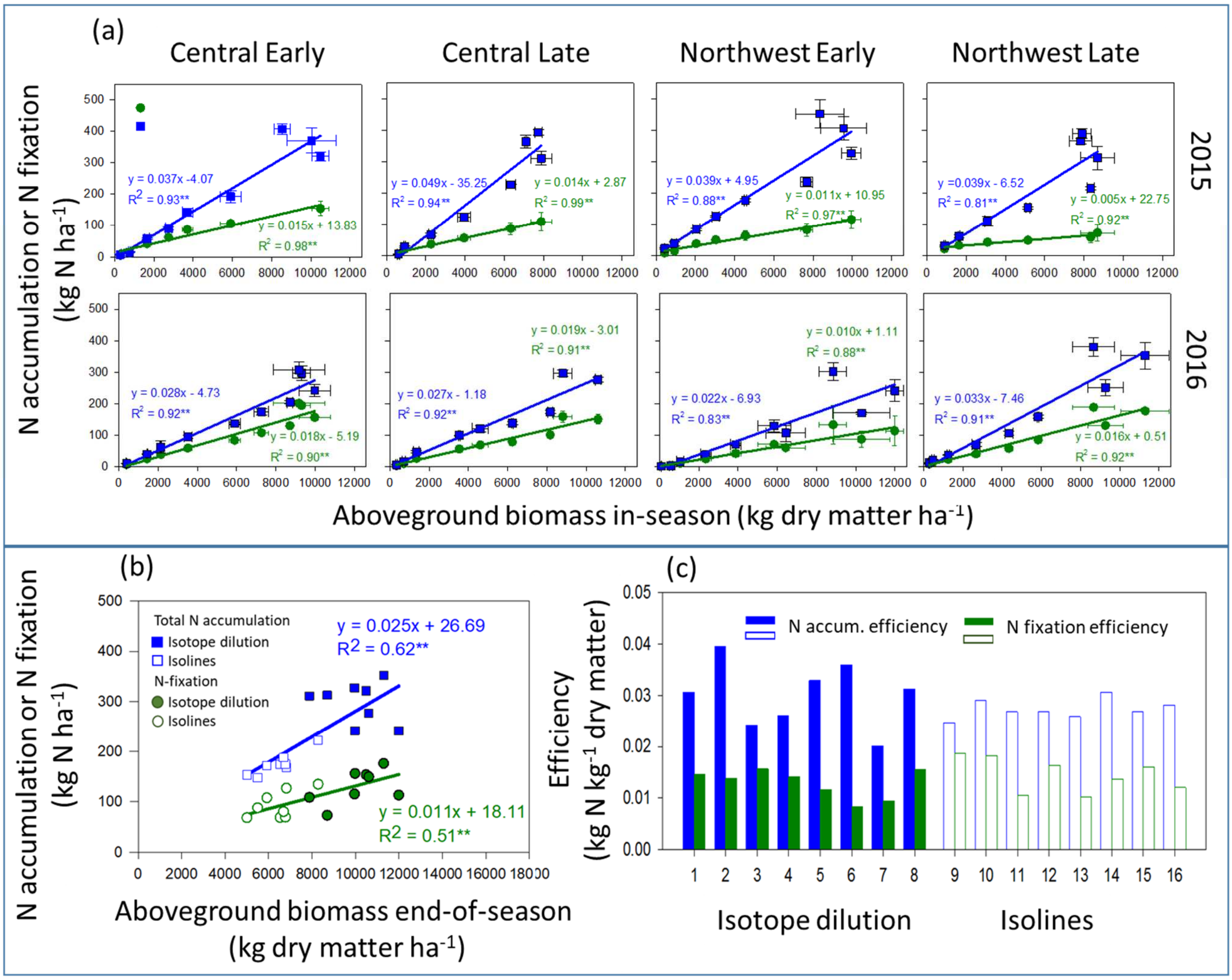

Figure 5. Top panels: In-season soybean aboveground N accumulation (blue squares) and aboveground BNF (green circles) as a function of aboveground biomass accumulation for isotope dilution experiment (a). Bottom left panel: End-of-season, at beginning of physiological maturity, soybean N accumulation and BNF as a function of biomass production for the isotope and isolines experiments (blue open squares for N accumulation, and green open circles for BNF) (b). Bottom right panel: Efficiency of N accumulation and BNF at beginning of physiological maturity for each experimental treatment (x-axis numbers correspond to treatment numbers listed in Table 1) (c).

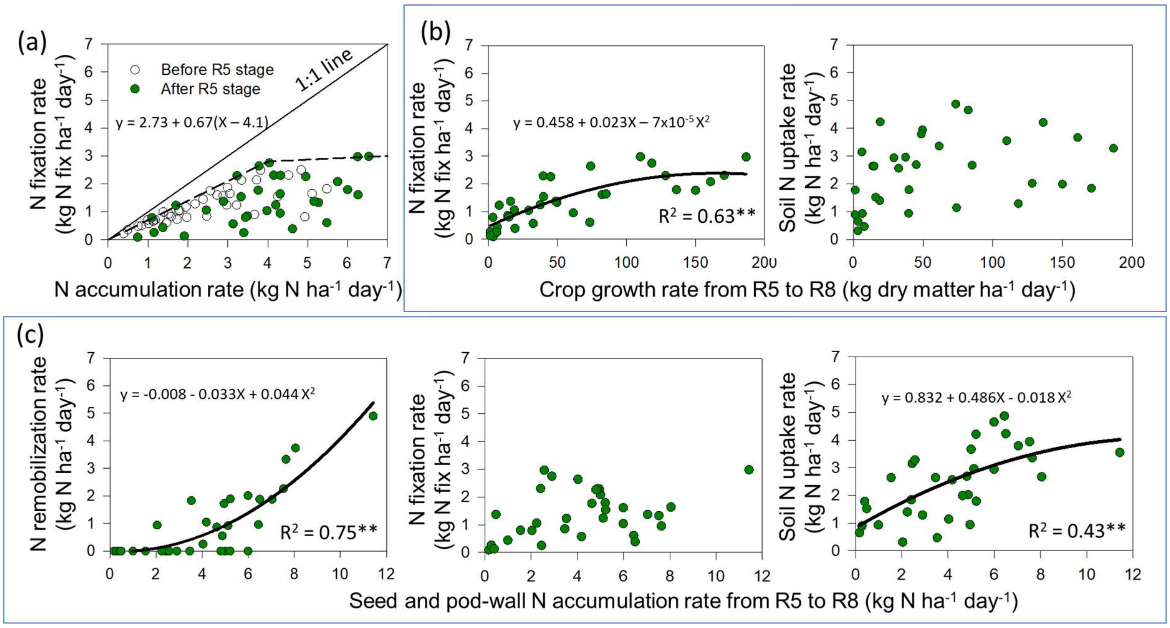

Figure 6. Top right panel: soybean aboveground BNF versus aboveground N accumulation rate (BNF and soil inorganic N uptake) before and after R5 stage (a). Top left panels: aboveground BNF and soil inorganic N uptake rate versus daily crop growth rate (carbon supply) during seed fill period (b). Bottom left panel: aboveground N remobilization, BNF, and soil inorganic N uptake rates versus seed and pod-wall N accumulation rate (N demand) during seed fill period, respectively (c). N remobilization was calculated as seed and pod-wall N accumulation rate minus the total aboveground plant N accumulation rate. Polynomial and bilinear fits illustrated in the above panels were significant at $p<0.001$. Values are means of three replications per year, site and planting date obtained from the isotope dilution experiment. The rates were calculated using the actual sampling dates and data derived from the logistic equation.

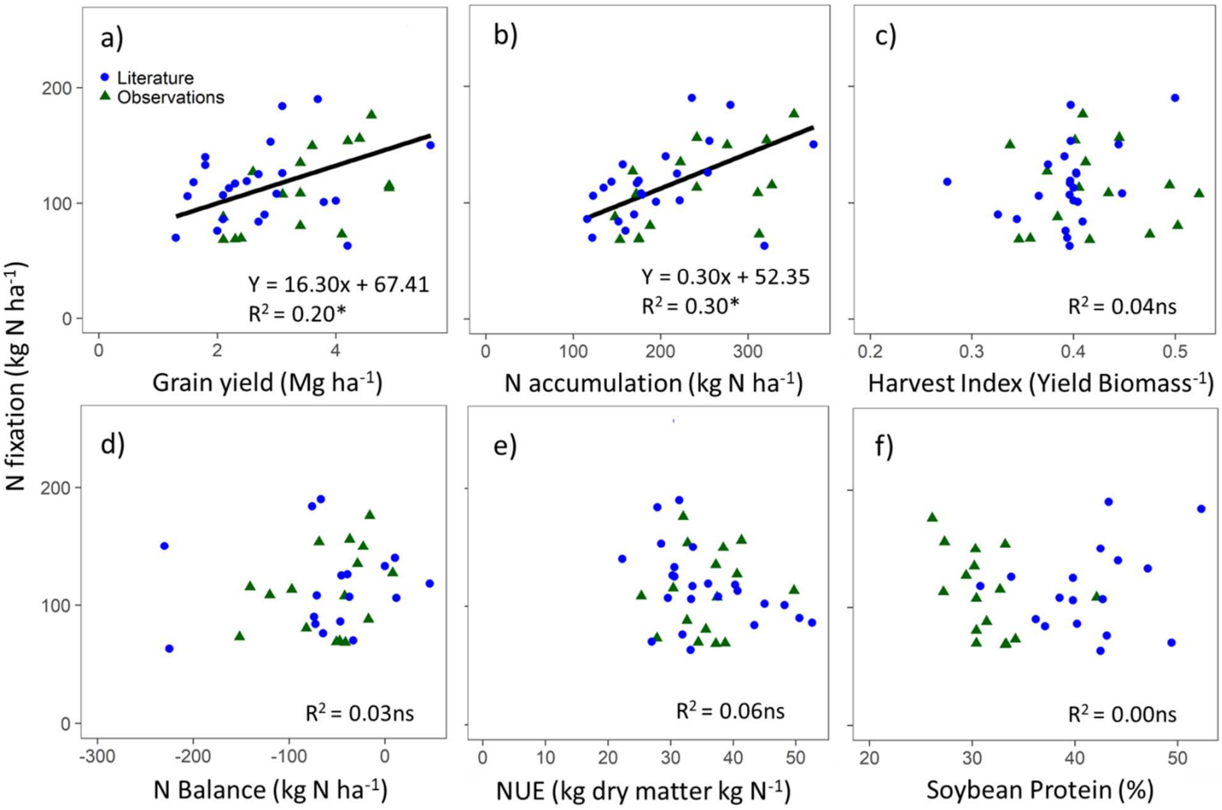

Figure 7. Top panels: Soybean yield (a), total aboveground biomass N accumulation (b), harvest index (c). Bottom panels: N balance (BNF minus seed N uptake; panel d), N use efficiency (e), and seed protein concentration (f) as a function of cumulative aboveground biomass BNF measured at the beginning of physiological maturity of of measured observations from both experiments isotope dilution and isolines (green triangles, data point is mean n=3) and literature observations (blue circles). Linear regression was fitted to both datasets, black solid line denoted statistically significance (p-value < 0.05).