A reassessment of the contribution of soybean biological nitrogen fixation to reactive N in the environment

Ilya Gelfand · G. Philip Robertson

Abstract The expansion of soybean (*Glycine max* (L.) Merr) acreage, increasing yields, and recent nitrogen (N) fertilization recommendations could have a major effect on the contribution of biological N fixation (BNF) in soybeans to reactive nitrogen (N_r) in the environment. We used ^{15}N natural abundance to separate fixed N into grain, aboveground vegetative biomass, and roots along a 9-point N-fertilizer gradient to ask: 1) is the belowground BNF contribution sufficiently different from aboveground to affect regional estimates of soybean N_r production based on harvested biomass, and 2) how does N fertilizer affect soybean yield and BNF's contribution to different tissues? The contribution of root and vegetative biomass to overall plant BNF was five times lower than that for grain. Including this difference in BNF extrapolations translates to 3.5 ± 0.5 Tg N_r yr^{-1} for total US soybean production, $\sim 37\%$ lower than earlier estimates that did not differentiate tissue source. Production of N_r ranged between 35 ± 11 and 73 ± 5 g Nr kg^{-1} grain and was affected by both fertilization and irrigation. In all cases N credits to the next rotational crop were minor. N-fertilization at even very low levels (17–50 kg N ha^{-1}) did not affect yield, but grain N content increased with fertilizer level. The percent BNF contributed to plant N decreased linearly with increasing fertilization, in grain from $49 \pm 8\%$ in unfertilized plots to a plateau of $16 \pm 6\%$ at fertilization ≥ 85 kg N ha^{-1}; in aboveground vegetative biomass from $77 \pm 4\%$ to a plateau of $11 \pm 11\%$ at 146 kg N ha^{-1}; and in roots from $88 \pm 12\%$ to a plateau of $41 \pm 6\%$ at 146 kg N ha^{-1}. The average whole-plant BNF contribution decreased from $\sim 84\%$ in unfertilized plots to a plateau of $\sim 34\%$ at fertilization rates greater than 84 kg ha^{-1}. Results underscore the unnecessary expense and environmental burden of adding N fertilizer to modern soybean varieties, and provide a refined lower estimate for the contribution of soybean N fixation to the US and global N_r budgets of 3.5 and 10.4 Tg Nr yr^{-1}, respectively.

Keywords Nitrogen uptake · ^{15}N natural abundance · Legumes · Roots · Yield response · Irrigation

I. Gelfand (✉) · G. Philip Robertson
W.K. Kellogg Biological Station, Michigan State University, Hickory Corners, MI 49060, USA
e-mail: ilya.gelfand@kbs.msu.edu

I. Gelfand · G. Philip Robertson
Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, MI 48824, USA

G. Philip Robertson
Department of Plant, Soil, and Microbial Sciences, Michigan State University, East Lansing, MI 48824, USA

Introduction

Soybean is an important agricultural crop with a large and expanding cropping area globally. In the U.S., for example, from 1990 to 2013 average US soybean yields increased by ~ 20 % from 2.2 Mg ha^{-1} yr^{-1} in 1990 to ~ 2.7 Mg ha^{-1} yr^{-1} in 2013, and acreage increased by ~ 36 % from $\sim 23 \times 10^6$ ha in 1990 to 31×10^6 ha in 2013 (USDA NASS 2014). Moreover, soybean agriculture has undergone rapid expansion around the globe; in Brazil, soybean cropped areas exceeded 25×10^6 ha in 2012, compared to less than 15×10^6 ha only a decade before (Alves et al. 2003; FAO 2007; Soystat 2013). The expansion of soybean acreage, together with increasing yields, could have significant implications for N$_r$ inputs to the environment (Martinelli et al. 2006) owing to the loss of newly fixed N into waterways, aquifers, and the atmosphere.

Estimates of N$_r$ production due to soybean cultivation are uncertain, although agricultural systems with BNF as a major source of new N tend to have lower N surplus and thus appear to lose less nitrate than systems with high N fertilizer inputs (Blesh and Drinkwater 2013; Syswerda et al. 2012). Current global estimates of N$_r$ loss from soybeans are based on reported yields, the grain to biomass ratio, and the contribution of BNF to total soybean biomass N. Four major assumptions are inherent: 1) the N content of non-grain above- and belowground biomass is 3 %; 2) the harvest index of soybean plants (the grain: total aboveground biomass ratio) is 0.4; 3) the ratio of aboveground N to total crop N content is 1.5; and 4) 68 % of total plant N is provided from BNF (N delivered from the atmosphere, or Ndfa) (Peoples et al. 1995a, b; Herridge et al. 2008). This last assumption is particularly uncertain because the contributions of belowground and aboveground vegetative biomass to total plant Ndfa and therefore to N$_r$ production can be much lower than that of grain. If so, N$_r$ production by soybeans might be significantly overestimated.

Moreover, the effect and fate of N fertilizer added to soybeans, currently recommended by the International Plant Nutrition Institute (2014) and others (Pioneer 2014; University of Minnesota 2014) to be added at a rate of ~ 50 kg N ha^{-1} for high yielding varieties based on evidence reviewed by Salvagiotti et al. (2008), is unknown. Is BNF reduced in the presence of fertilizer N as theory predicts or does moderate fertilization, especially during early development,

increase Ndfa over the full growing cycle (van Kessel and Hartley 2000; Ruiz Diaz et al. 2009)?

Altogether, these uncertainties lead to a very wide range of estimates of the contribution of BNF by crop legumes to the global N cycle (e.g., Smil 1999; Galloway et al. 2004; Herridge et al. 2008), with estimates ranging between 10 and 32 Tg N$_r$ yr^{-1}. Compared to natural, pre-industrial N fixation of ~ 58 Tg N yr^{-1} (Vitousek et al. 2013) this range represents between 20 and ~ 50 % increase in the global N$_r$ pool due to soybean BNF.

Many studies provide estimates of the contribution of BNF to aboveground soybean biomass (including both grain and shoots), which, on average, accounts for ~ 60 % of total soybean N uptake (Salvagiotti et al. 2008). This generalized estimate of the contribution of BNF to the total N budget of soybeans has been reported across a range of environmental and soil conditions. A few studies, however, have reported higher rates of up to 80 % in Brazil (Alves et al. 2003) and Argentina (e.g., Herridge et al. 2008), where soybeans have been bred on unfertilized soils.

By contrast, assessments of belowground BNF contributions (root Ndfa) are sparse and lower. The contribution of belowground BNF to the whole plant N balance at maturity range from ~ 24 to ~ 40 % for irrigated and dryland soybeans in Australia (Rochester et al. 1998; Unkovich and Pate, 2000). By including Australian estimates of belowground fixed N, Herridge et al. (2008) updated the ~ 10 Tg N$_r$ yr^{-1} estimate by Smil (1999) to 16.4 Tg N$_r$ yr^{-1}.

Soybeans can also meet their N needs from soil inorganic N pools. Because BNF is energy intensive (Silsbury 1977), soybeans are expected to favor inorganic N uptake when N availability in soil increases (Peoples et al. 1995b; Schipanski et al. 2010). This expectation for a changing contribution of BNF to the aboveground biomass has been tested in field studies. Using the ^{15}N natural abundance method, Schipanski et al. (2010) showed a quadratic-like decrease in the BNF contribution with an increase in soil N availability in fields under legume vs. fertilizer N management. In a direct fertilization experiment, with the addition of between 0 and 180 kg N ha^{-1} in four increments, Goss et al. (2002) showed a linear decrease in BNF with increasing N fertilization. After reviewing over 50 studies over the past 40 years in which N fertilizer was added (ranging between 0 and 1,800 kg N ha^{-1}),

Salvagiotti et al. (2008) drew a highly scattered negative exponential relationship between fertilization and the total fixed N in soybean aboveground biomass. We are aware of no studies that have examined the differential responses of above versus belowground Ndfa to increasing N fertilization rates.

At least two questions remain: 1) is the belowground contribution to soybean Ndfa sufficiently different from the aboveground contribution to affect regional estimates of soybean N_r production based on harvested biomass; and 2) how does N fertilizer affect soybean yield and the Ndfa in different plant parts? We investigated these questions at a site in SW Michigan, USA, where we fertilized a modern commercial transgenic soybean variety at nine different N levels ranging from 0 to 146 kg N ha^{-1}. We further compare rates of BNF in an irrigated non-fertilized check plot and in a conventionally managed tilled system that received starter fertilizer of 9 kg N ha^{-1}.

Materials and methods

Experimental site and design

The experimental site is located in SW Michigan in the northeast portion of the U.S. Corn Belt, at the W.K. Kellogg Biological Station (KBS) Long-Term Ecological Research (LTER) site (www.lter.kbs.msu.edu; Robertson and Hamilton 2015) (42°24′N, 85°24′W at 288 m elevation). The mean annual air temperature at KBS is 9.7 °C and annual rainfall averages 900 mm yr^{-1}, evenly distributed seasonally with about half falling as snow. Soils are well-drained Typic Hapludalfs developed on glacial outwash. During the experimental year (2012), the summer was drier than usual with 226 mm of total precipitation between soybean planting (22 May) and harvest (9 October) compared to a long-term average of 369 mm (NOAA 2009; lter.kbs.msu.edu/datatables/7). Daily temperature averages ranged between 13.4 ± 5.0 (minimum) and 27.1 ± 5.8 °C (maximum).

The experimental plots are part of the LTER resource gradient experiment established in 2005 (Robertson and Hamilton 2015). The plots are 4.6 × 27.4 m arranged randomly in eight blocks (four rainfed and four irrigated). The plots received standard potassium (K$_2$O) and phosphorus (PO$_4^-$) fertilization (130 kg K ha^{-1} and 73–123 kg P ha^{-1} based on soil tests), with

lime also applied as needed (2–5 Mg ha^{-1}) to moderate soil pH. All plots are in the same corn-soybean-wheat rotation. Although soybeans are normally not differentially fertilized in this rotation, for this experiment soybeans were treated with nine N fertilization levels: 0, 17, 34, 50, 67, 84, 101, 123, and 146 kg N ha^{-1} as granular ammonium-nitrate (38-0-0; treatments F0–F146, respectively) on 14 June, prior to the R1 development stage. None of the plots were tilled.

We examined BNF in all rainfed N fertilization treatments and in the unfertilized irrigated treatment. Additionally, we examined BNF in four replicate 1 ha plots under conventional management (i.e., chemical inputs and soil preparation) within the KBS LTER Main Cropping System Experiment (MCSE; Robertson and Hamilton 2015). The conventionally managed plots in the MCSE received 9 kg N ha^{-1} as part of a starter NPK fertilizer application per normal farming practices in Michigan.

Plant sampling and analysis

We planted in all experimental plots a commercially available soybean variety (Pioneer 92Y30 RR) and for reference five soybean plants of a non-nodulating isoline (PI 547695, seed source: 04U-3266; Horosoy) obtained from the USDA Soybean Germplasm Collection (USDA-ARS, Urbana, IL). The nodulating plants were sown using standard field equipment; to sow the non-nodulated plants we manually removed commercial seeds from soil and exchanged them with non-nodulating seeds.

Ten plants (5 nodulating and 5 non-nodulating) were harvested by hand at full seed stage (R6.5). We harvested whole plants, together with the majority of coarse and fine roots, by collecting all the soil within a 25 × 25 cm wide × 25 cm depth soil volume. Soil was carefully shaken from the roots in the field, and the roots were examined for the presence or absence of nodules. Then the roots were placed on a 4 mm screen and washed with sprinkled water to remove soil. All plants from the same replicate plot were composited. Plant material was then divided into three major components: grain, aboveground vegetative biomass (stem, leaves, and pods less seeds) and belowground biomass (roots including nodules). These three components represent different pathways of N_r flow: grain is removed from the field and used for food/feed; aboveground vegetative biomass is dispersed across

the field during harvest; and belowground biomass decomposes in place in the field.

Plant components were oven dried to a stable weight at 60 °C and then weighed and ground to pass a 200 μm mesh sieve. Then subsamples were weighed in duplicate into tin cups and sent for ^{15}N natural abundance and total N content analysis (Stable Isotope Facility, UC Davis, USA).To correct for percent ash in samples we measured ash by burning subsamples in a muffle furnace at 550 °C for at least 4 h and weighed the residue as total ash.

Nitrogen fixation estimation

We used the ^{15}N natural abundance method (Schipanski et al. 2010) to estimate BNF in soybeans. The percentage of BNF in the N-fixing (nodulated; N_{fix}) soybean (Pioneer) biomass was calculated using the non-nodulating (nonN) soybeans as reference plants:

$$\%N \text{ fixation} = 100 \times \left[\left(\delta^{15}N_{nonN} - \delta^{15}N_{Nfix} \right) / \left(\delta^{15}N_{nonN} - B \right) \right] \quad (1)$$

where $\delta^{15}N_{nonN}$, and $\delta^{15}N_{Nfix}$ are $\delta^{15}N$ values of nodulating and non-nodulating plants, respectively and B is the $\delta^{15}N$ value of soybean grown with atmospheric N as the only source of N. The total fixed N in each component of the plant was calculated from the dry matter, N concentration, and %N from fixation.

To quantify B, we grew in N-free media in the greenhouse 24 plants of the commercial varieties Pioneer 92Y30 RR and Blue Rive Hybrids Certified organic (OCIA) in 12 pots containing a mixture of sand, Turface MPV (Profile products, Buffalo Grove, IL, USA), and perlite; seven pots had Pioneer and five pots had Blue River hybrids. We inoculated each pot with a 10 ml slurry made of a bulked soil sample taken prior to planting from all experimental plots and combined with distilled water in a 1:3 proportion. The plants were fertilized with N-free Hoagland's nutrient solution (0-7-5 NPK with micronutrients; GreenCare Fertilizers, Chicago, IL, USA). The plants grown in the greenhouse were harvested and analyzed for $\delta^{15}N$ in the same way as the field plants.

Yield and N balance estimation

For yield determinations soybeans in all plots were harvested on 9 October using standard field equipment.

For estimating the effect of water limitation on soybean yields we compared yields of non-irrigated plots to irrigated plots in the current year and to non-irrigated plots in 2009. In 2009, the precipitation during the soybean growing season was 382 mm (compared to 226 mm for the 2012 growing season) and the minimum and maximum average daily temperatures were 12.2 ± 4.0 (mean $\pm$ S.D.) and 24.4 ± 4.6 °C, respectively, as compared to 13.4 ± 5.0 and 27.1 ± 5.8 °C in 2012.

To calculate N_r production due to soybean cultivation in the US we determined the average plant population density for the conventionally managed treatment at harvest ($329 \times 10^3 \pm 18 \times 10^3$ plants ha^{-1}). Then we used Ndfa values for the conventional treatment, except for roots. For roots we substituted the average Ndfa value for F0 and F17 (Fig. 1c).

The N balances for experimental treatments were calculated as the difference between the sum of N from the soil ($Soil_N$) and N fixation (BNF_N) and harvested N ($Harvest_N$) (Eq. 2). For the calculation of N_r, we added N inputs from fertilization ($Fert_N$) and BNF_N (Eq. 3), and to calculate the N credit, we subtracted harvested N from N_r (Eq. 4):

$$N \text{ balance} = [Soil_N + BNF_N] - Harvest_N \quad (2)$$

$$N_r = BNF_N + Fert_N \quad (3)$$

$$N \text{ credit} = [BNF_N + Fert_N] - Harvest_N \quad (4)$$

To calculate N balance, N_r, and N credit for the F84 treatment we substituted root BNF_N with the roots Ndfa from F34 to F123 (Fig. 1c).

Results

Contribution of BNF to N content in grain, aboveground, and belowground biomass

All reference plants grown in the greenhouse showed nodulation and exhibited very similar $\delta^{15}N$ values irrespective of the commercially available cultivar: grain, -1.50 ± 0.05 ‰ ($\pm$ standard error, $n = 12$ pots); aboveground vegetative biomass, -2.78 ± 0.10 ‰ ($\pm$ standard error, $n = 12$); belowground biomass, 5.30 ± 0.29 ‰ ($\pm$ standard error, $n = 11$). The nitrogen contents of these plants were: grain, 5.79 ± 0.12 % ($\pm$ standard error, $n = 12$); aboveground
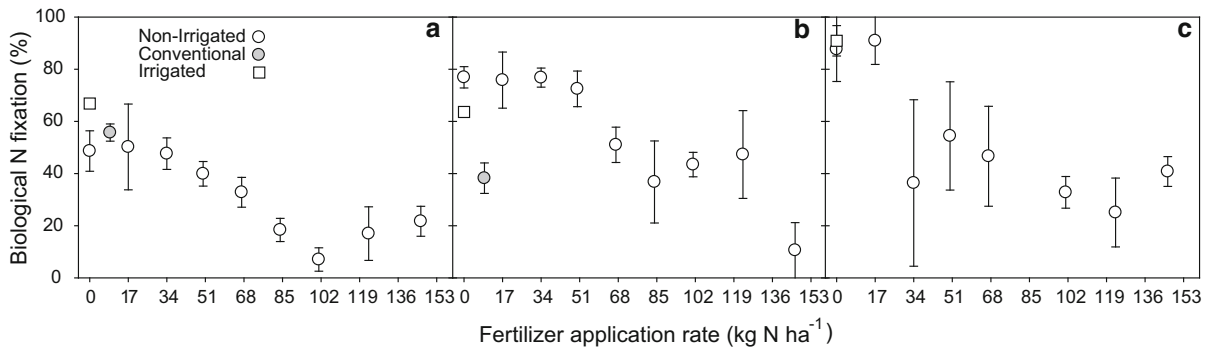
Fig. 1 Contribution of BNF (%) to the N content of **a** harvested grain, **b** aboveground vegetative biomass, and **c** roots of soybean plants as a function of the N fertilizer application rate in non-irrigated no-till plots (*open circles*), Conventionally managed plots including tillage and starter fertilizer (*shaded circles*), and Irrigated and unfertilized no-till plots (*open squares*) (mean ± s.e. $n = 4$)

vegetative biomass, 1.80 ± 0.11 % ($\pm$ standard error, $n = 12$); and belowground biomass, 2.49 ± 0.07 % ($\pm$ standard error, $n = 11$).

Biological nitrogen fixation contributed 49 ± 8 % to the N content of grain in all rainfed treatments with no N fertilizer and as little as 22 ± 6 % at high fertilizer levels (Fig. 1a; 146 kg N ha^{-1}). In the irrigated treatment BNF contributed 67 ± 2 % to total N content of the grain. The contributions of N fixation to grain N content exhibited a linear decrease with fertilizer N level, reaching a plateau of 16 ± 6 % at fertilization levels between 84 and 146 kg N ha^{-1} (Fig. 1a). Over all fertilization levels, the relative contribution of grain N originated from BNF to total plant Ndfa averaged 84 ± 3 % (Table 1).

In the aboveground vegetative (non-grain) portions of rainfed plants, the contribution of BNF to N content exhibited a different pattern. BNF contributed ~ 80 % to the total vegetative N content in treatments fertilized at rates between 0 and 50 kg N ha^{-1}, ~ 50 % in treatments fertilized between 67 and 123 kg N ha^{-1}, and reached a minimum of ~ 11 % at the application rate of 146 kg N ha^{-1} (Fig. 1b). Biological N fixation contributed between 60 and 40 % to the aboveground vegetative biomass in the unfertilized irrigated plots and in the conventionally managed plots, respectively (Fig. 1b). The relative contribution of aboveground vegetative biomass N originated from BNF to total plant Ndfa averaged 12 ± 2 % (Table 1).

The BNF contribution to the total N content of roots exhibited a linear decrease with increasing fertilization rates. Over all treatments, BNF contributed to total root N between $\sim 91 \pm 6$ % in the unfertilized

plots and 41 ± 6 % at 120 kg N ha^{-1} (Fig. 1c). However, the relative contribution of root biomass N originated from BNF to total plant Ndfa averaged 4 ± 1 % (Table 1).
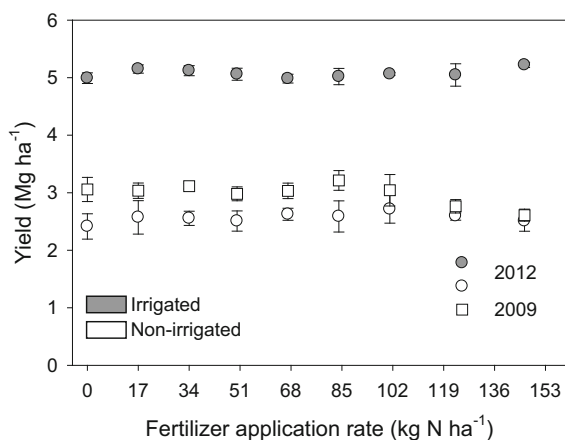
In rainfed treatments, the shoot to root ratio was 2.3 ± 0.1, while in irrigated treatments the shoot to root ratio was 4.9 ± 0.3. Overall, the grain to total biomass ratio (including belowground biomass) was 1.0 ± 0.0, and the harvest index (grain to aboveground biomass ratio) was 0.5 ± 0.0 in all studied treatments.

Yield and N content of plants in response to fertilization and irrigation

Measured yields exhibited no response to fertilization; average of non-irrigated plots 2.6 ± 0.1 Mg grain ha^{-1} (Fig. 2). Irrigated plots yielded 5.1 ± 0.1 Mg grain ha^{-1} also with no response to fertilization (Fig. 2). In contrast, grain N content exhibited a linear increase with increasing N fertilization (Fig. 3). Grain N content in non-nodulated plants significantly increased from 4.9 ± 0.5 to 7.0 ± 0.2 % N of grain dry weight as N fertilization rates increased from 0 to 146 kg N ha^{-1}. Grain N content in nodulated plants exhibited a smaller but significant linear increase from 5.8 ± 0.6 to 6.4 ± 0.1 % (Fig. 3). Irrigation also had an effect on the N content of grain in nodulated plants, which increased from 5.8 ± 0.5 to 6.5 ± 0.4 % and in non-nodulated plants from 4.9 ± 0.5 to 6.2 ± 0.2 %, for rainfed and irrigated plants, respectively. On the other hand, the N contents of vegetative biomass and roots were not affected by fertilization or irrigation in either

Table 1 Proportion of N delivered from N_2 fixation (Ndfa) in soybean plants incorporated into aboveground vegetative biomass, roots, and grain
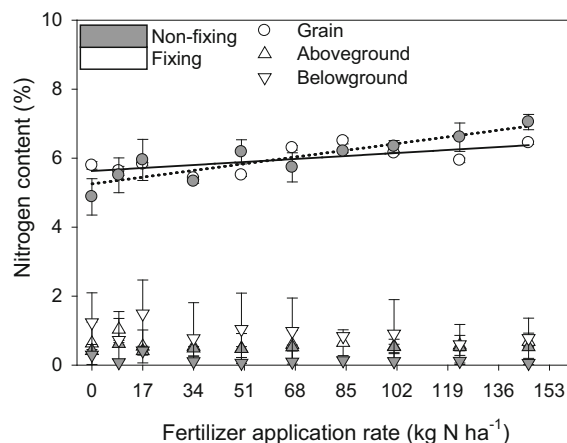
Treatment	Fertilizer application rate $kg\ N\ ha^{-1}$	Aboveground vegetative biomass %	Roots	Grain
F0	0	11	5	84
F17	17	8	5	87
F34	34	12	1	87
F50	50	10	4	86
F67	67	8	3	89
F84	84	13	5	82
F101	101	34	9	57
F123	123	14	4	81
F146	146	2	0	98
Conventional	0	9	6	86
Irrigated	9	12	3	85
Average		12 ± 2	4 ± 1	84 ± 3

Fig. 2 Yield response to fertilization and irrigation during the 2012 growing season (*open and shaded circles*) and comparison to non-irrigated yields in 2009 when soybeans were not fertilized (*open squares*) (mean ± s.e. $n = 4$)

Fig. 3 N content of in different plant tissues as a function of N fertilizer rate (average ± s.e., $n = 48$). Full symbols represent non-fixing plants. For non-fixing plants (*dotted line*), $R^2 = 0.797$, $p = 0.0005$; and for fixing plants (*solid line*), $R^2 = 0.445$, $p = 0.0351$

non-nodulated or nodulated plants, with N contents of 0.7 ± 0.1 and 0.9 ± 0.1 % of dry weight, respectively.

N balance, N credit, and N_r

Soybean N balance, calculated as the difference between N removed in harvested grain and the sum of N acquired from soil and BNF (Eq. 2), was close to zero and not affected by fertilization in any fertilized treatment. Balances ranged from -29 ± 29 kg N ha^{-1} for plots fertilized with 101 kg N ha^{-1} to 41 ± 24 kg N ha^{-1} for plots fertilized with 34 kg N ha^{-1}

(Table 2). The largest positive balance of 41 ± 24 kg N ha^{-1} was calculated for plots that received 34 kg N ha^{-1} (Table 2). The conventional MCSE and irrigated treatments, on the other hand, exhibited highly positive balances of 76 ± 16 and 171 ± 45 kg N ha^{-1}, respectively. Nitrogen credits for the next rotational crop calculated as the difference between N removed in harvested grain and the sum of N acquired from BNF and N applied as fertilizer were negative or very low in all treatments (Table 2).

The total production of N_r, which includes N from BNF and fertilizer, ranged from an average of

Table 2 Effect of N fertilization on the soybean plants' N relationships, N balance, and reactive N production (including error propagation) in no-till fertilized, no-till irrigated, and conventionally managed treatments

Treatment	N fertilizer application rate $Kg\ N\ ha^{-1}$	Biological N fixation	N Uptake from Soil	Harvest N	N balance	N credit	N_r	Yield weighted N_r $g\ N_r\ kg^{-1}$
F0	0	84 (27)	68 (9)	140 (20)	13 (35)	−55 (33)	84 (27)	35 (11)
F17	17	94 (30)	69 (14)	150 (21)	14 (39)	−38 (36)	111 (30)	43 (12)
F34	34	88 (13)	90 (16)	137 (12)	41 (24)	−15 (17)	122 (13)	48 (5)
F50	50	59 (10)	82 (19)	139 (13)	3 (25)	−29 (17)	109 (10)	44 (4)
F67	67	60 (18)	115 (22)	165 (13)	9 (31)	−39 (22)	127 (18)	48 (7)
F84	84	32 (9)	145 (76)	168 (20)	9 (79)	−52 (22)	116 (9)	45 (4)
F101	101	10 (3)	128 (24)	167 (16)	−29 (29)	−56 (16)	111 (3)	41 (2)
F123	123	39 (23)	149 (28)	154 (9)	34 (38)	8 (25)	162 (23)	62 (9)
F146	146	37 (10)	147 (22)	162 (13)	22 (28)	21 (17)	183 (10)	73 (5)
Irrigated	0	283 (25)	158 (19)	269 (33)	171 (45)	13 (41)	283 (25)	57 (5)
Conventional[a]	9	80 (11)	69 (7)	73 (9)	76 (16)	16 (15)	89 (11)	47 (6)

[a] Yield weighted N_r from soybean BNF (without fertilization) is equal to $43 \pm 6\ g\ N_r\ kg^{-1}$

~ 112 kg N ha^{-1} for N application rates between 0 and 101 to 162 ± 23 and 183 ± 10 kg N_r ha^{-1} for application rates of 123 and 146 kg N ha^{-1}. The irrigated treatment produced 283 ± 25 kg N_r ha^{-1} and the conventional treatment 89 ± 11 kg N_r ha^{-1} (Table 2). Yield-weighted N_r production exhibited very little variation, with an average production of 45 ± 2 g N_r kg^{-1} harvested grain in all but the treatments with 123 and 146 kg N ha^{-1} application rates, where yield-weighted production was 62 ± 9 and 73 ± 5 g N_r kg^{-1} grain (Table 2).

Discussion

Root and aboveground vegetative biomass contributed substantially less BNF (Ndfa) than grain to total N_r production by soybeans. The root and aboveground vegetative biomass, altogether, contributed 16 % to N_r production at a zero fertilization level, while grain contributed 84 %. At the plant scale, BNF contributed 50, 80, and 90 % to the total N content in grain, aboveground vegetative biomass, and roots, respectively (Fig. 1). The discrepancy between the Ndfa of specific parts of plant and the relative contribution of plant parts to total N_r production is due to the low N content of aboveground vegetative biomass and roots, and a high shoot to root ratio. Therefore, while BNF contributed a significant amount of N to the aboveground and belowground vegetative biomass N

balances, the total soybean N_r production was most affected by Ndfa associated with the grain.

Soybean yield was not affected by fertilization; similar results have been shown for other cultivars including Brazilian (Alves et al. 2003). Fertilization, instead, had an effect on the contribution of BNF to plant N, which decreased with added fertilizer N. We found, however, that increasing fertilization positively affected the N content of the grain, but not that of aboveground vegetative biomass and roots (Figs. 2, 3). Similarly, the decrease in BNF contribution's to plant N balance due to increasing fertilization was reflected in overall neutral N balances at all fertilization levels, which translates to little or no N credits to the next rotational crop (Table 2). Finally, moderate fertilization during early development had no effect on Ndfa over the full growing cycle in contrast to earlier suggestions of increased Ndfa from early fertilization (i.e., van Kessel and Hartley 2000; Ruiz Diaz et al. 2009).

Overall, fertilization (a) reduced the amount of N fixation, (b) did not contribute to additional yield, and (c) above a modest level of added N, contributed to the environmental N_r burden.

Effect of N fertilization on BNF in soybeans

The contribution of BNF to the N content of grain, aboveground vegetative, and belowground parts of soybean plants was different under different fertilization

levels. The contribution of BNF to grain N decreased linearly from ~ 50 % with no N fertilizer to ~ 16 % at fertilization between 84 and 146 kg N ha^{-1} (Fig. 1a). The contribution of BNF to the vegetative aboveground biomass N content also decreased with increasing fertilization, from 80 % at fertilization between 0 and 50 kg N ha^{-1} to ~ 10 % at 146 kg N ha^{-1} (Fig. 1b). The contribution of BNF to root N content showed a pattern similar to grain as it decreased linearly from ~ 90 % BNF contribution at zero fertilizer towards a plateau of ~ 30 % contribution at fertilization levels between 101 and 146 kg N ha^{-1} (Fig. 1c). The relative importance of the three plant parts to the total Ndfa of the soybean plants averaged 84, 12, and 4 percent for grain, aboveground, and belowground parts, respectively, and showed no clear response to fertilization (Table 1).

While our estimates of the average proportion of BNF contributions to the grain N content are similar to those found by others (i.e., Salvagiotti et al. 2008; Schipanski et al. 2010; c.f. Alves et al. 2003), our estimates of the BNF contribution to belowground and aboveground vegetative biomass N and contribution of these plant parts to total soybean Ndfa are significantly different from the reported values of 24–40 % for roots and 60 % for aboveground residuals (Herridge et al. 2008; Rochester et al. 1998; Unkovich and Pate 2000) currently used for global N budgets calculations.

In contrast to the recommended application rate of ~ 50 kg N ha^{-1} (i.e., International Plant Nutrition Institute 2014) and earlier occasional reports of a yield response to N fertilizer application (e.g., Gan et al. 2003; Heatherly and Elmore, 2004; Salvagiotti et al. 2008), we found no yield response to N fertilizer in either rainfed or irrigated treatments (Fig. 2). What might explain this lack of response? Since BNF is an energy and carbon (C) demanding process with an average cost of 6 g C per 1 g fixed N (Vance and Heichel 1991), one might expect that when plants avoid this cost they would be able to allocate more carbon and energy to reproductive tissue (i.e. grain). Two explanations seem plausible: First, there may have been an additional limiting factor at our site other than N, P, K, and water. This possibility is unlikely, however, since standard agronomic soil tests at our site have not noted any trace element deficiencies (http://lter.kbs.msu.edu/datatables/354). Second, energy intensive N compounds may have accumulated in grain without contributing to the production of grain

mass. If this were the case, the grain N content of the N-fixing plants would have increased, which was, in fact, the case: grain N content increased from 5.8 ± 0.1 to 6.4 ± 0.1 % with increasing levels of fertilization (Fig. 3).

The grain N response of the non-fixing soybean plants to N fertilization was much stronger than that of the N-fixing plants (Fig. 3). The grain N content of the non-fixing plants steadily increased with increased N fertilization levels by more than 2 %, from 4.9 ± 0.5 to 7.1 ± 0.2 % N. This can be explained by the interplay between the additional carbon cost of BNF and N availability. Under high fertilization levels the N-fixing plants, which cannot completely down regulate resource-intensive BNF (Silsbury 1977), maintained N fixation rates of ~ 16 % despite high soil N availability, and accumulated less N in grain at the highest fertilization level as compared to non-fixing plants (6.4 vs. 7.1 %; Fig. 3).

Even with high yields during a more favorable climate year our soybeans would have been unlikely to have responded to fertilizer N. Yields of 6.7 Mg ha^{-1} (100 bu acre^{-1}) would require a total N uptake of ~ 380 kg N ha^{-1}; based on this study a minimum of 51 % of this total would have been satisfied by BNF, leaving ~ 190 kg N ha^{-1} to be provided by soil organic matter mineralization, which is within the mineralization supply of these moderately fertile loams (Millar and Robertson 2015).

Effect of N fertilization and irrigation on N$_r$ produced by soybean cultivation

Fertilization rates of up to 101 kg N ha^{-1} affected N$_r$ production by soybean, which ranged between 84 ± 27 and 127 ± 18 kg N$_r$ ha^{-1} mainly due to fertilizer inputs. In highly fertilized and irrigated treatments, N$_r$ production was also high, due to high N fertilizer inputs rather than increased grain delivered Nr, except in the irrigated treatment. When scaled by grain yield there were no differences among treatments except at the highest fertilization levels. The estimated N$_r$ production in the MCSE conventionally managed soybean crop was 89 ± 11 kg N$_r$ ha^{-1} and is similar to the 111 kg N$_r$ estimated by Salvagiotti et al. (2008) despite a relatively dry growing season during our experiment.

During the growing season (June–October) rainfall at our site was 226 mm or 60 % of normal precipitation.

Yields were consequently depressed by 14 % on average (range of 4–21 %; Fig. 2) as compared to 2009, when rainfall (382 mm) was slightly more than the long-term growing season average of 369 mm for our site (NOAA 2009; lter.kbs.msu.edu/datatables/7). The drier conditions and lower yields in 2012 could potentially lead to an underestimation of N_r production. However, they did not seem to have a major effect on the BNF contribution to soybean N balance (i.e., Fig. 1).

Regional perspective

Based on an average US soybean production between 2002 and 2012 (FAOSTAT 2014; 2.8 ± 0.1 Mg ha^{-1}), which was very similar to soybean yield at our site for 2012 (2.6 ± 0.1 Mg ha^{-1}), and our estimate of N_r from the conventionally managed plots, we can calculate the total N_r delivered to the environment due to soybean cultivation. Reactive N production from the conventionally tilled plots in 2012 was 69 kg N_r ha^{-1} in grain, 7 kg N_r ha^{-1} in aboveground vegetative biomass, and 4 kg N_r ha^{-1} in roots. Total N_r production due to soybean BNF therefore sums to 80 ± 11 kg N_r ha^{-1} (excluding fertilization; Table 2). This translates to 43 ± 6 g N_r kg^{-1} grain (Table 2). US soybean production between 2002 and 2012 averaged 82×10^6 $\pm 2 \times 10^6$ Mg (USDA NASS 2014; FAOSTAT 2014), which translates to 3.5 ± 0.5 Tg of new N_r.

Our estimate of N_r based on the differential contribution to whole soybean plant Ndfa by different plant parts (i.e., grain, aboveground vegetative biomass, and roots) is ~ 37 % lower than the 5.7 Tg N_r estimated for US soybean production by Herridge et al. (2008) based on 2005 yields. The difference stems from a) their assumption of 60 % Ndfa for grain, aboveground biomass and roots of soybeans versus our different percentages for different parts of the plant (Fig. 1, Table 2), b) their assumption of 3 % N in parts of the plants other than grain versus our ~ 1 % N as measured in the field, and c) their estimate of total crop biomass N based on multiplying shoot N by a factor of 1.5, which translates to an estimated contribution of belowground biomass N to the overall soybean N budget of 50 % versus our measured contribution of ~ 4 % (Table 1).

Current global estimates of N_r from BNF, calculated by Herridge et al. (2008) are 16.4 Tg N. If we assume similar tissue differentiation as for this study, the global estimate is likely overestimated by 37 %, such that global N fixation from soybean BNF is likely

10.4 Tg N_r. This value is about ~ 18 % of terrestrial BNF prior to human alternation of the N cycle (Vitousek et al. 2013).

Conclusions

Nitrogen fertilization across a wide range of fertilizer levels did not affect soybean yields but resulted in higher grain N content and diminished biological N fixation. Above modest fertilizer levels, grain N content increased with no further attenuation of N fixation, leading to higher N_r production.

Fertilization affected the N content of plant tissues differently: in grain, fertilizer decreased the percent of N fixed from 49 to 16 %, in aboveground vegetative biomass from 77 to 11 %, and in roots from 88 to 41 %. Overall, fertilizer N reduced N fixation in soybean plants from 84 to 34 %.

The contribution of root and vegetative biomass to total plant N accumulation was ~ 20 % of that for grain. Extrapolating tissue-specific values to US soybean production provides an estimate of 3.5 ± 0.5 Tg of new N_r added to the environment annually, ~ 37 % lower than prior estimates that did not differentiate tissue source.

Our overall results underscore the general futility of adding N fertilizer to modern soybean varieties, even in starter amounts, and as well its additional cost to environmental Nr loading.

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