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UNDERSTANDING NITROGEN LIMITATION IN SOYBEAN

by

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UNDERSTANDING OF NITROGEN LIMITATION IN SOYBEAN

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Meeting soybean demand on existing cropland area for a global population of 9.7 billion people by the year 2050 requires narrowing the existing gap between average producer yield and yield potential. Soybean relies on two sources on nitrogen (N): biological N_2 fixation and indigenous soil N supply. As soybean yield continues to increase, it seems critical to know if there is a yield level at which potential contribution of indigenous nitrogen sources and fixation becomes insufficient to meet crop N requirements for high yields, while still maintaining or increasing protein and oil concentration. This study evaluated N limitation across 29 high-yield soybean environments in Argentina and Nebraska from 2015 to 2017. Each environment included a ‘zero-N’ treatment, which forced the crop to rely on biological N_2 fixation and indigenous soil N, and a ‘full-N’ treatment, which provided an ample fertilizer N supply during the entire crop cycle based on novel protocol developed also in this study. Seed yield and protein concentration in full N were 11% and 3% higher than zero-N, respectively. The magnitude of the difference depended upon the yield level of the production environment, ranging from 0 kg ha^{-1} at 2.5 Mg ha^{-1} up to 900 kg ha^{-1} at 6 Mg ha^{-1}. Seed yield responses were directly related with increases in accumulated N in aboveground biomass (70 kg N ha^{-1}), without changes in nitrogen use efficiency. The N limitation was mitigated in environments with large contribution of indigenous soil N supply. The maximum rates of N limitation occurred before the seed filling and the plant mechanisms and processes underlying seed yield and protein concentrations were leaf area index, absorbed solar radiation, and N remobilization. Finally, there was a trade-off between biological N_2 fixation and indigenous soil N supply with fixation reduced less than proportional per unit increase in indigenous N sources. There was a temporal asynchrony between biological N_2 fixation and N demand, that is, biological N_2 fixation was not sufficient to meet plant N demand as the latter increased and the contribution of indigenous soil N supply decreased. The peak of indigenous soil N supply was the most important factor explaining variation in the N limitation across environments. Findings from this study will help to narrow soybean yield gap to meet future food demand.

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CHAPTER 1: SOYBEAN NITROGEN LIMITATION IN THE CONTEXT OF INCREASING GLOBAL FOOD DEMAND

1.1 Soybean production and uses

Soybean is the most cultivated legume in the world accounting, for 56% of total global oilseed production (Wilson et al., 2008). Soybean total production and harvest area are 319 MMT and 119 million ha, respectively (FAOSTAT, 2015-2017; http://www.fao.org/faostat/en/#data/TP). Main producing countries are the United States (USA), Brazil, and Argentina, accounting for respective 34, 30, and 17% of global production. East Asia (mostly China and Japan), European Union, South-East Asia, and Mexico are the major importing countries (FAOSTAT, 2012-2016; http://www.fao.org/faostat/en/#data/TP). Soybean production has increased during the last 40 years due to increases in harvest area and seed yield. Annual yield gain has ranged from 29 to 38 kg ha^{-1} y^{-1} due to crop breeding, improved agronomic practices, and the corresponding interaction between both factors (Specht et al., 2014; Grassini et al., 2014).

Soybean seed contains ca. 19% oil (used for cooking, biodiesel and other industrial uses), 68% meal fraction (used as animal feed) and 13% water (http://www.nopa.org/resources/datafacts/soybean-composition/#). The summation of 68% in the meal fraction comprises 36% protein, 19% fiber, 9% soluble carbohydrates, and 4% of minerals; taking these proportions as a relative weight of the whole seed. The soy meal fraction can be processed for other products such as soy milk, tofu, and flour. This combination of oil and protein content makes soybean unique among world's major crops. However, this uniqueness comes along with high nutrient requirements and nitrogen (N) is indeed one of the most demanded nutrients in soybean.

1.2. Nitrogen in soybean: sources and requirements

Nitrogen is an essential macronutrient as it is part of chlorophyll pigments that have an important functional role on capturing energy from sunlight. The major nitrogen-containing compound in leaves is the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). This enzyme is a catalyzer in the photosynthetic assimilation of CO_2. Therefore, N concentration in leaves is directly related to maximum photosynthetic rate that leaves can achieve. Storage N in the leaves has also been found to be a main driver of leaf expansion (Liu et al., 2018). This N pool in leaves (and also in stems and petioles) is mobilized to the seeds during seed filling to meet seed N demand. The N remobilization can reduce leaf N concentration and consequently accelerate leaf senescence and reduce leaf photosynthesis during seed filling (Sinclair and Muchow, 1999).

On average, soybean crops accumulate ca. 80 kg N ha^{-1} in the aboveground biomass for each metric ton of seed yield produced (Salvagiotti et al., 2008; Tamagno et al., 2017; Figure 1-1). It is about 3 times greater than the N requirement per unit of grain in cereal crops such as rice, wheat, or maize (Setiyono et al., 2011; Yin et al., 2019; Barraclough et al., 2010). Therefore, the soybean N uptake requirement can be very high, especially in high-yield production environments. For example, the average soybean yield of 3 Mg ha^{-1} obtained over the last 5 years in the USA and Argentina, (USDA-NASS, 2013-2017; https://www.nass.usda.gov/Quick_Stats/; https://datos.magyp.gob.ar/) requires ca. 240 kg N uptake ha^{-1}. In contrast, the N uptake requirement in the most favorable production environments of the US Corn Belt ranges from 480 -640 kg N ha^{-1} in order to produce seed yields ranging from 6 to 8 Mg ha^{-1} (Specht et al.; 1999; Grassini et al., 2014;Sinclair and Rufty; Van Roekel and Purcell).

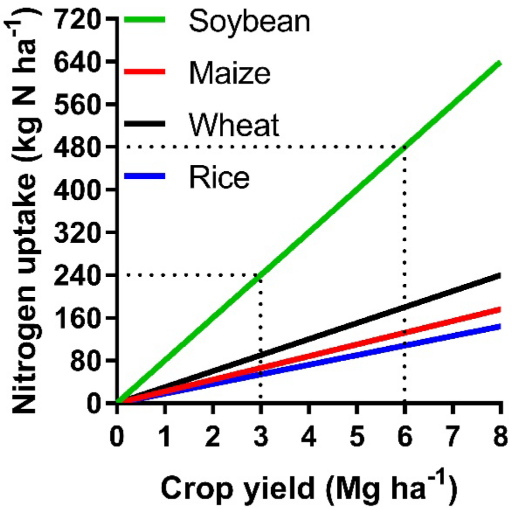

Figure 1-1. Crop nitrogen requirement per metric ton of yield produced for cereal crops (maize, wheat, and rice) and soybean. The crop nitrogen requirement was calculated from the accumulated N in aboveground biomass measured at physiological maturity. Dotted lines indicate the N requirement associated with different soybean yields.

In contrast to cereal crops, fertilizer N is rarely used on soybean, except for a small amount (< 20 kg N ha^{-1}) sometimes applied as 'starter' at sowing. Hence, soybean relies on biological N_2 fixation and other sources of N including soil N supply from organic matter mineralization, dry and wet atmospheric deposition, N from irrigation water, and a small amount of starter N fertilizer (hereafter collectively termed 'indigenous soil N supply'). A negative association (trade-off) between N_2 fixation and indigenous soil N supply has been documented in the literature (Streeter and Wong, 1988). This trade-off occurs because soybean preferentially acquires N from soil than from N fixation owing to the associated energetic cost of N fixation (Connor et al., 2011). As soybean yield continues to increase due to breeding and agronomic management (Specht et al., 2014), the question arises as to whether biological N_2 fixation and indigenous soil N supply will be sufficient to meet the crop N requirements in current and future high-yielding production scenarios.

1.3. Research justification and goals

Meeting soybean demand on existing cropland area for a global population of 9.7 billion people by the year 2050 requires narrowing the existing gap between average producer yield and yield potential (Cassman et al., 2003; van Ittersum et al., 2013). Yield potential is defined as the yield of a well-adapted cultivar when grown without limitations in water and nutrient supply and kept free of biotic stresses (weeds, diseases, and insect pests) (Evans, 1993). Increasing soybean yield to close the yield gap has an associated increase in crop N requirements that has to be fulfilled either from indigenous sources or biological N_2 fixation. Moreover, seed protein concentration is negatively correlated with seed yield and it is unclear whether this negative correlation is, in part, related to N supply or not (Chung et al., 2003; Rincker et al., 2014). Considering that there is an upper limit to the amount of N that can be provided by the soil, it seems critical to know whether N fixation is enough to sustain high seed yield and maintain seed protein concentration. Hence, the goal of Chapter 2 is to determine whether soybean yield and seed protein concentration are limited by N supply or not across a wide range of yield levels (from 2.5 to 6.7 Mg ha^{-1}). Chapter 3 aims to understand the if seed yield and protein concentration increases due to ample N supply are related to changes in total N uptake, N-use efficiency (NUE), N harvest index (NHI), and/or the indigenous N supply in high-yield environments. Chapter 4 identifies the physiological mechanisms underlying in-season N limitation in soybean. Chapter 5 studies the possible causes for N_2 fixation not being able to fill the gap between indigenous soil N supply and plant N demand in high-yield soybean crops analyzing the dynamics of in season N demand, biological N_2 fixation and indigenous soil N supply. An overall discussion of the thesis is presented in Chapter 6 and future research priorities are highlighted.

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CHAPTER 2: IS SOYBEAN YIELD LIMITED BY NITROGEN SUPPLY?

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Abstract

As soybean yield continues to increase, it seems critical to know if there is a yield level at which potential contribution of indigenous nitrogen (N) sources (N fixation and soil mineralization) becomes insufficient to meet crop N requirements for high yields, while still maintaining or increasing protein and oil concentration. We have hypothesized that, in absence of other limiting factors, degree of N limitation increases with increasing yield potential (Yp) of the production environment. To test this hypothesis, we developed a novel protocol to ensure an ample N supply during the entire crop season (full-N treatment). That protocol was applied to field-grown soybean in Balcarce (Argentina) and Nebraska (USA), where measured full-N seed yields were ±15% of their simulated Yp in 92% of the cases. The combination of locations, years, sowing dates, and N treatments resulted in a wide range of seed yields, from 2.5 to 6.5 Mg ha^{-1}. Overall, full-N seed yield averaged 11% higher than seed yield without N addition (zero-N). However, magnitude of yield difference between full-N and zero-N depended upon Yp, ranging from no detectable yield difference in low-Yp (ca. 2.5 Mg ha^{-1}) to up to 900 kg ha^{-1} in high-Yp environments (ca. 6 Mg ha^{-1}). Seed yield differences were associated with higher aboveground dry matter, seed number, and seed weight in the full-N versus zero-N treatments. Seed protein (but not oil) concentration was higher in the full-N treatment, and both protein and oil yields were higher in the full-N versus zero-N treatments. Findings from this study indicate that (i) N limits soybean seed yield (as well as protein yield, and oil yield) in environments with high Yp, where indigenous N sources seem insufficient to fully satisfy crop N requirements, and (ii) yield response to N fertilizer can occur above a 2.5 Mg ha^{-1} Yp threshold and has upper limit of 250 kg seed per Mg increase in Yp.

Keywords: Soybean, nitrogen, yield potential, protein, oil

2.1. Introduction

Soybean [Glycine max (L.) Merr.] is the most important legume crop globally, with a respective harvested area and total production of 118 million ha and 307 million Mg (FAOSTAT 2014, http://faostat3.fao.org), accounting for 56% of total global oilseed production (Wilson et al., 2008). Soybean is a key component of global food security as a source of protein for human food and animal feed, and oil for cooking and biofuel. Meeting soybean demand on existing cropland area for a global population of 9.7 billion people by year 2050 will put pressure on narrowing the existing gap between average producer yield and yield potential (Cassman et al., 2003; van Ittersum et al., 2013). Yield potential (Yp) is defined as the yield of a well-adapted cultivar when grown without limitations in water and nutrient supply and kept free of biotic stresses (weeds, diseases, and insect pests) (Evans, 1993). Hence, for a given site-year, soybean Yp is determined by solar radiation, temperature, and other factors that influence the length of time during which the crop was grown, such as variety maturity group and sowing date. For example, soybean Yp has been postulated to range between 6 and 8 Mg ha^{-1} in favorable environments of the US Corn Belt (Specht et al., 1999; Sinclair and Ruffy, 2012).

Relatively high amounts of nitrogen (N) must be taken up by all crops to achieve high seed yields, particularly legumes, because of their high seed protein content (Sinclair and de Wit, 1979; Giller and Cadisch, 1995). On average, a soybean crop accumulates ca. 79 kg N ha^{-1} in its aboveground biomass per each additional Mg seed yield, with the latter expressed at standard seed moisture of 0.130 kg H_2O kg^{-1} seed (Salvagiotti et al., 2008; Tomagno et al., 2017). This ratio can be used to estimate the N uptake requirement over a range of soybean seed yields. For example, seed yields ranging from 6 to 8 Mg ha^{-1} would be expected to have an associated N uptake requirement range of 480 to 640 kg N ha^{-1}. In contrast, only 240 kg N uptake ha^{-1} would be required for a soybean yield of 3 Mg ha^{-1}, which is equivalent to average soybean yield during the last 5 years in the United States (US) and Argentina (USDA-NASS, 2010-2014; https://www.nass.usda.gov/Quick_Stats/; https://datos.magyp.gob.ar/). Soybean rarely receives N fertilizer in producer fields (though a small application as ‘starter’ fertilizer is sometimes applied at sowing time). Still, there is currently much interest in the degree to which well-managed soybean crops, grown in favorable production environments, can meet the large N requirement for 6 to 8 Mg ha^{-1} seed yields by relying exclusively on soil N mineralization and N fixation.

Soybean yield continues to increase over time due to genetic and agronomic improvement (Grassini et al., 2014; Specht et al., 2014). Hence, it would be useful to discern the seed yield level at which the potential contributions of N from fixation and N from soil organic matter mineralization might jointly become insufficient to satisfy crop N requirement. Acquisition of that information requires data collection from soybean crops grown in stress-free conditions, where measured yields approach the site-year-specific Yp, as determined by weather, sowing date, and variety. In such scenarios, experimental comparison of a ‘zero-N’ treatment receiving no N fertilizer (i.e., crop must rely exclusively on the indigenous N sources including N-fixation) versus a full-N treatment receiving N fertilizer applied as needed to sequentially ensure ample N supply throughout the crop-growing season. This comparison would need to be repetitively conducted across a wide range of Yp production systems to generate sufficient data for determining whether there is a yield level at which those indigenous N sources are insufficient to meet crop N requirements.

Meeting crop N requirement is challenging because it requires temporal synchronization between the seasonal supply of N from indigenous N sources and seasonal crop N demand, and addition of N fertilizer when the latter exceeds the former at any time during the crop season (Cassman et al., 2002). No previous study has explicitly attempted to grow soybean in production settings of very high yields while ensuring non N-limiting conditions (Salvagiotti et al., 2008 and references cited therein). While a few studies have reported using large N fertilizer amounts in soybean (>300 kg ha^{-1}), N fertilizer in these studies was applied as a single large dose near the sowing date, or in split applications during early vegetative stages (e.g., Brevedan et al., 1978; Herridge and Brockwell, 1988; Ray et al., 2006; Wilson et al., 2014). Because soybean absorbs ca. 60% of total N uptake during the pod setting and seed filling phases (Thies et al., 1995; Bender et al., 2015, Gaspar et al., 2017), it is difficult to determine the degree to which these previous studies have ensured non-N limiting conditions during those phases. Moreover, the yield response (or lack of response) to N fertilizer, reported by these previous studies, was likely confounded by other non-N growth-limiting factors. For example, in water-limited conditions, the yield response to N fertilizer can be amplified by the negative effect of temporary water shortages on N fixation (Purcell et al., 2004; Ray et al., 2006). Moreover, even in absence of water limitation, other growth-reducing factors may have limited crop growth in these experiments, given that measured yields consistently fell short of the high range of 6 to 8 Mg ha^{-1} soybean Yp (Specht et al., 1999, Sinclair and Ruffy, 2012), and also were less than measured yields (5-6 Mg ha^{-1}) that are routinely attained by progressive soybean producers (Grassini et al., 2014, 2015).

In this study, we hypothesized that, in absence of other limiting factors, the degree of N limitation increases with higher Yp. To test this hypothesis, we developed a protocol to ensure ample N supply during each phase of the soybean crop season. The protocol was applied to field-grown irrigated crops in Balcarce (Argentina) and Nebraska (USA) that were within ±15% of their simulated Yp based on site-year specific weather, sowing date, and variety. Results were interpreted using simple eco-physiological frameworks.

2.2. Materials and methods

2.2.1. Field experiments

Field experiments were conducted in Balcarce (BA), Argentina, during two crop seasons (BA-Y1: 2014/2015 and BA-Y2: 2015/2016), and at four sites in Nebraska (NE), USA during one crop season (2016). The experiments in BA consisted of a combination of sowing date, variety maturity group, and N treatments (zero-N and full-N, see Section 2.2), whereas experiments in NE were replicated at four producer irrigated high-yield fields that included the same two N treatments (Table 2-1). For simplicity, the combinations of crop season x sowing date x variety in BA, or the producer fields in NE, are hereafter called ‘environments’. In all experiments, crops were irrigated and managed to ensure optimal water and nutrient supply (except, of course, for N in the zero-N treatment, see Section 2.2) and to avoid stress from weeds, insects, and pathogens. Irrigation was applied throughout the crop season with application event amounts adjusted periodically to match seasonal changes in crop water demand. In NE producer fields, soil water content in the upper 90 cm of soil was monitored using Watermark® sensors, which indicated that soil water status was consistently above 65% of total soil plant available water between emergence and physiological maturity. Several prophylactic foliar applications of herbicide, fungicide, and insecticide kept the crops free from biotic stresses in all experiments. A meteorological station located at each site provided daily weather data.

2.2.2. Balcarce, Argentina

Field experiments were conducted on a deep fine-loamy Typic Argiudol. Topsoil (0-20 cm) organic matter, extractable phosphorous (P Bray-1 method), and soil pH were 27 g C kg^{-1}, 22 mg kg^{-1}, and 6.4 in BA-Y1, and 28 g C kg^{-1}, 25 mg kg^{-1}, and 6.6 in BA-Y2. Soil $N\text{-}NO_3^-$ in the upper 60 cm at sowing ranged from 59 to 114 kg ha^{-1} in BA-Y1 and from 61 to 105 kg ha^{-1} in BA-Y2. Previous crop was wheat and hairy vetch [Vicia villosa Roth.] in BA-Y1 and BA-Y2, respectively. The field was disked before sowing only in BA-Y1. Seeds were treated with fungicide and inoculated in both years using the best available product in the market and following recommended inoculation practices (Nitragin Optimize II® Pack Apron® & Jumpstart). Row spacing was 0.35 m and plants were thinned at V1 stage to ca. 35 plants m^{-2}. Phosphorous, sulfur, and calcium fertilizer amounts applied before sowing were 19, 21, 26 kg ha^{-1} in BA-Y1 and 20, 22, 27 kg ha^{-1} in BA-Y2, respectively.

Table 2-1. Description of field experiments conducted in Balcarce (Argentina) and Nebraska, NE (USA).

Experiment	Crop season	Location	Variety name and maturity group (MG)	Sowing date
Balcarce (BA-Y1)	2014/2015	37.7647 S 58.3125 W 118 m a.s.l.	DM2200 (MG:2.1), DM3810 (MG:3.8), DM4612 (MG:4.6),	Nov 1, Nov 27, Dec 18, Jan 6
Balcarce (BA-Y2)	2015/2016	37.7652 S 58.3117 W 118 m a.s.l.	DM2200 (MG:2.1), DM3312 (MG:3.3), DM3810 (MG:3.8),	Nov 3, Oct 12, Jan 11
Atkinson, NE	2016	42.6372 N 98.9561 W 635 m a.s.l.	AG2723 (MG:2.7)	April 25
Mead, NE	2016	41.2441 N 96.5016 W 368 m a.s.l.	AG2723 (MG:2.7)	May 8
Saronville, NE	2016	40.6005 N 97.9658 W 538 m a.s.l.	AG2431 (MG:2.4)	April 26
Smithfield, NE	2016	40.5380 N 99.6833 W 769 m a.s.l.	P24T19 (MG:2.4)	May 13

The factorial experiments in Argentina were arranged in a split-split plot design with four replicates in both years. Sowing dates were main plots, three varieties of contrasting maturity group were subplots, and the two N treatments were sub-subplots (Table 2-1). Sub-subplot size was 3.8 x 10 m and 6.3 x 6 m for BA-Y1 and BA-Y2, respectively. Sowing dates were equally spaced by 200°Cd (BA-Y1) and 300°Cd (BA-Y2) based on measured daily temperature and using a base temperature (T_b) of 10°C (Tenorio et al., 2017). The purpose of combining different maturity groups and sowing dates was to generate a wide range of Yp along which the two N treatments could be compared. On average, there was a yield decrease of 38 kg ha^{-1} per day of delay in sowing, resulting on a yield difference of ca. 2.6 Mg ha^{-1} between earliest and latest sowing date treatments.

2.2.3. Nebraska, USA

Experiments were conducted in four pivot-irrigated producer fields in NE located near Atkinson, Mead, Saronville, and Smithfield during the 2016 crop season (Table 2-1). These fields had persistently produced high soybean yields in previous years (>5.0 Mg ha^{-1}). Soils were deep, without physical or chemical constrains to root growth. Soil series were O'Neil sandy loam (Atkinson), Yutan silty clay loam (Mead), Hastings silt loam (Saronville), and Holdrege silt loam (Smithfield). Average initial soil tests in the upper 30 cm indicated a pH of 6.3, 6.1, 6.1, and 7.2; organic matter of 9, 20, 18, and 16 g C kg^{-1}; extractable P (Bray-1 method) of 49, 16, 126, and 16 mg kg^{-1}; and 1 M NH_4-acetate extractable K of 357, 335, 395, and 487 mg kg^{-1}. Soil N-NO_3^- in the upper 60 cm at sowing ranged from 33 to 54 kg ha^{-1} across locations. The previous crop was maize in the four fields. Fields were disked (Atkinson and Saronville), strip-tilled (Mead), and no-till

(Smithfield). In all four fields, row spacing was 0.76 m, and seeding rate ranged from 35 to 45 seeds m^{-2}, well above those recommended to maximize seed yield (De Bruin and Pedersen, 2009). Seeds were treated with fungicide and insecticide in all cases, but received no inoculant, which is the common producer practice for fields with a prior crop history of soybean in the US Corn Belt, primarily because of the lack of yield response to inoculation, especially in high-yield environments (De Bruin et al., 2015; Leggett et al., 2017 and references cited therein). Pre-sowing nutrient applications (in kg ha^{-1}) included N (20), P (22), K (50), Ca (62), Mg (8), S (17), Zn (2) at Atkinson, N (13) P (20) S (11) Zn (1) at Mead, N (17), P (35), S (10), Zn (1) at Saronville, and P (38), S (1) at Smithfield. Experiments in each field were arranged in a completely randomized design with two N treatments, and four replicates per treatment. The eight contiguous plots (16 x 11 m each) were purposely located in a high-yield area in each field, which was identified based on field yield maps from previous years.

2.2.4. Protocol for nitrogen fertilizer application

There were two N treatments (full-N and zero-N), which were randomly assigned to the sub-subplots (BA) or to the replicates (NE). The aim of the full-N treatment was to ensure non-N limiting conditions during the entire crop season by temporal additions of N fertilizer. In contrast, the zero-N treatment relied on indigenous N sources, including inorganic soil N at sowing, in-season N mineralization, and N fixation. Crops also received a small N ‘starter’ application at sowing (<20 kg N ha^{-1}) in three of the four producer fields in NE. Similarly, crops in NE received additional (non-fertilizer) groundwater N input through irrigation (57, 1, 7, and 2 kg N ha^{-1} in Atkinson, Mead, Saronville, and Smithfield, respectively). At each site, contribution from indigenous N sources, excluding N fixation, was estimated by measuring total N uptake in the aboveground dry matter at physiological maturity of a maize crop grown in a zero-N plot located adjacent to each experiment.

The protocol developed to apply N fertilizer in the full-N treatment was designed to fully satisfy the crop N requirements needed to support the Yp calculated for each environment. Soybean CROPGRO model (Boote et al., 1998), embedded in DSSAT v 4.5 (Jones et al., 2003; Hoogenboom et al., 2010), and SoySim model (Setiyono et al., 2010) model were used to simulate Yp in BA and NE, respectively, using long-term (>25 years) measured daily weather data. These two models have been satisfactorily evaluated on their ability to reproduce measured yields in well-managed experiments (Setiyono et al., 2010; Aramburu Merlos et al., 2015). Maximum simulated Yp across years was ca. 7.2 (early sowing) and 3.8 Mg ha^{-1} (late sowing) in BA and ca. 7.5 Mg ha^{-1} for all four sites in NE. The latter is consistent with the Yp for soybean of 6 to 8 Mg ha^{-1} proposed by Specht et al. (1999) and Sinclair and Ruffy (2012) for favorable production environments in the US Corn Belt.

Total N fertilizer amount to use in the full-N treatment was calculated based on the maximum Yp simulated for each environment (crop season x sowing date x variety in BA and producer field in NE), and a seed yield-N uptake ratio of 12.7 kg kg^{-1} (Salvagiotti et al., 2008; Tomagno et al., 2017) (Figure 2-1, inset). No attempt was made to account for belowground N (including N in roots, nodules, exudates, and rhizodeposition) because this parameter is likely to exhibit large variation across the wide range of environmental conditions evaluated in our study. Contribution from N fixation and mineralization were not accounted for the calculation of N fertilizer amount, because of the uncertainty in N supply from soil mineralization and trade-offs between N fixation and applied N fertilizer (Streeter, 1988). Total N fertilizer amount was increased by 40% in the four NE fields to compensate for potential N losses through volatilization and leaching resulting from mismatches between irrigation or rainfall events and fertilizer application and a few heavy rainfall events (>50 mm) that occurred early in the season. In contrast, irrigation was applied right after the two largest N applications at R3 and R5 in BA, without occurrence of heavy rainfall events; hence, N fertilizer amount was increased only by 10%. Total N fertilizer amount applied in the full-N treatment ranged from 330 to 640 kg N ha^{-1} across sowing dates in BA and averaged 870 kg N ha^{-1} in NE producer fields.

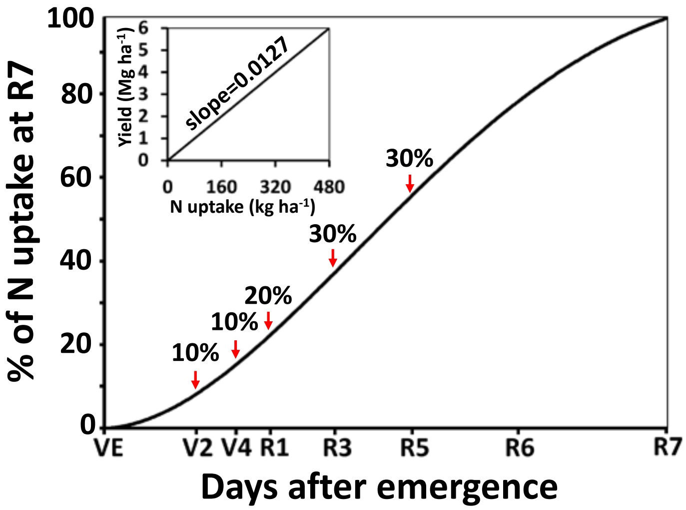

Figure 2-1. Scheme showing the nitrogen (N) fertilization protocol followed in the experiments. Total N fertilizer amount was estimated for each environment based on site-specific yield potential and the relationship between seed yield and total N uptake (inset). Total N fertilizer amount was split into 5 applications (red arrows) based on the temporal (daily basis) dynamics of N uptake in soybean (solid line). As shown, the N fertilizer amount applied in each application corresponded to 10% (V2 and V4 stages), 20% (R1 stage), and 30% (R3 and R5 stages) of total applied N fertilizer. Crop stages are based on Fehr and Caviness (1977) staging system and are spaced on the x-axis according to the approximate calendar date of their occurrence in the experiments.

Total N fertilizer was split in several applications during the crop season as a means of synchronizing the N supply and crop N demand (Figure 2-1). Based on seasonal N uptake patterns reported by Thies et al. (1995) and Bender et al. (2015), total N fertilizer amount was split in five applications (V2, V4, R1, R3, and R5 stages), with respective N fertilizer amounts representing 10%, 10%, 20%, 30%, and 30% of the total applied N fertilizer.

Granular urea fertilizer was applied and manually broadcasted between plant rows. The protocol was followed consistently in all experiments (BA) and producer fields (NE).

2.2.5. Measurements

Phenological events were tracked on a weekly basis in all experiments following the Fehr and Caviness (1977) staging system. Abscised leaves were collected weekly starting at R3 using nets placed in-between rows. At physiological maturity (R7 stage), 1-m row of consecutive plants in each sub-subplot in BA, or replicate in NE, surrounded by two rows receiving the same N treatment, was clipped at the soil surface and oven-dried to constant weight at 70°C. Aboveground dry matter (ADM) at R7 was calculated as the sum of the standing dry matter plus the cumulative sum of abscised-leaf dry matter. The ADM samples were threshed by hand, then divided into the seed and non-seed fractions. Harvest index (HI) was determined as the ratio between seed biomass (on an oven-dry matter basis) and ADM. Larger plant samples (1.8, 2.8, and 4.6 m^2 in BA-Y1, BA-Y2, and NE, respectively) were collected from each sub-subplot in BA or main plot in NE to obtain an end-of-season estimate of seed yield. Two sub-samples of 200 seeds each were weighed to estimate mean individual seed mass (weight basis), which was then used with sample yield to derive the number of seed per harvested area. Seed yield and seed weight were adjusted at 0.130 kg H_2O kg^{-1} seed. Soybean seed protein and oil concentration (in units of kg constituent kg^{-1} seed) were determined for each N treatment seed sample using near-infrared analysis (FOSS Infratec^{TM} 1241). Protein and oil yields (Mg ha^{-1})

were calculated by multiplying sample seed yield by the corresponding sample seed protein and oil concentrations. The yield and concentration of protein and oil were expressed on an oven seed dry-matter basis.

2.2.6. Data analysis

Agreement between measured yields and simulated Yp based on site-year-specific weather, sowing date, and variety maturity group was evaluated by calculating the root mean square error (RMSE) and absolute mean error (ME) as follows:

$$\mathrm{RMSE} = \sqrt{\frac{\sum(\mathrm{Y_P} - \mathrm{Y_M})^2}{\mathrm{n}}} \qquad \text{Eq. (1)}$$

$$\mathrm{ME} = \frac{\sum(\mathrm{Y_P} - \mathrm{Y_M})}{\mathrm{n}} \qquad \text{Eq. (2)}$$

where $\mathrm{Y_P}$ is the simulated yield potential and $\mathrm{Y_M}$ is the measured yield (Mg ha^{-1}). Separate RMSE and ME were calculated for the two N treatments (full-N and zero-N). Regression analysis was used to detect departure from the null hypothesis of unity in charts comparing the two N treatments with respect to yield and other measured traits.

A combined analysis of variance (ANOVA) was conducted to determine the effect of N treatments on seed yield, ADM, HI, seed number and weight, and seed protein and oil concentration and yield (SAS® PROCMIXED v.9.3) (Moore and Dixon, 2015). The combined analysis assumes homogeneity of within-environment variances. Our experiments were not too different in relation with their variances ($F_{max}<6$); hence, our combined ANOVA can be considered robust (Milliken and Johnson, 2009). In this analysis, each combination of crop season, sowing date, variety, and location was referred to as an environment. Each environment had two N treatments (full-N and zero-N), with four replicates. Environment and N treatments were treated as fixed effects. This analysis provided an estimate of the overall significance of the N treatment across a diverse range of Yp. Although environments (site x crop season x variety x sowing date) cannot be directly compared, the interaction between environment and treatment can be used to evaluate the consistency of the N treatment response.

Differences in seed yield between full-N and zero-N treatments were investigated using two simple physiological frameworks:

Seed yield (Mg ha^{-1}) = seed number (seed ha^{-1}) x mean seed weight (Mg seed^{-1}) Eq. (3)

Seed yield (Mg ha^{-1}) = ADM (Mg ha^{-1}) x HI Eq. (4)

Mean values calculated for the different parameters (seed yield, seed number and weight, ADM, HI, and seed protein and oil yields and concentration) for the full-N treatment were plotted against averages computed for the zero-N treatment. Departures from the

1:1 line (which represents the null hypothesis of no response differential between full-N and zero-N) were investigated using linear and quantile regression (quantreg package, R Development Core Team, 2016). To assess changes in the seed constituents of protein and oil in relation with increasing yields, linear and quadratic equations were fitted to the relationships between protein and oil yield versus seed yield.

2.3. Results

2.3.1. Seed yield as influenced by nitrogen treatments

Diversity of locations, crop season, sowing dates, varieties, and N treatments resulted in a wide range of soybean yield across environments, from 2.52 to 6.46 Mg ha^{-1} (Figure 2-2). A yield threshold of 4.5 Mg ha^{-1} has been used in the literature to define high-yield soybean environments (Salvagiotti et al., 2008). About half of measured yields in the present study were above this threshold. In fact, our highest yields fell well above the range reported in the literature for field-grown soybean in experiments with N fertilizer addition (see Salvagiotti et al., 2008 and references cited therein).

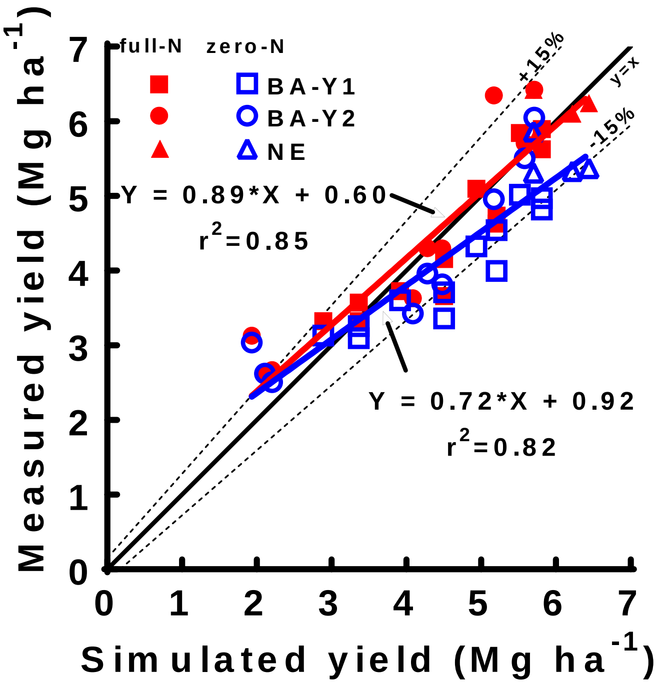

Figure 2-2. Measured yields versus simulated yield potential for the two treatments of zero-N (empty symbols) and full-N (solid symbols). Each data point corresponds to the simulated yield potential (Yp) and measured average yield for a given sowing date x variety x N treatment (Balcarce, BA) or for a producer field x N treatment (Nebraska, NE). Experiments were conducted in BA over two crop seasons (BA-Y1 and BA-Y2) and at four sites in NE during one crop season. Parameters of the fitted linear regression models (solid lines) are shown. Yp was simulated using on-site daily measured weather and actual sowing date, plant density, and variety maturity group.

In 92% of the cases, measured full-N yields were within ±15% of the simulated Yp values generated with well-validated crop models that were supplied with inputs of local measured daily weather data and based on treatment-specific sowing date, plant density, and variety maturity group (Figure 2-2). The two crop models (CROPGRO in BA and SoySim in NE) used here to simulate Yp performed better at reproducing measured yields in the full-N than in zero-N treatment, as indicated by their respective RMSE (0.51 versus 0.67 Mg ha^{-1}) and ME values (0.10 versus -0.36 Mg ha^{-1}). Moreover, the test of observed slopes versus the null hypothesis of slope=1 was non-significant for full-N ($P=0.20$), but was significantly different for the zero-N treatment ($P<0.001$) (i.e., the respective red and blue regression lines in Figure 2-2). Given that finding, one can reasonably infer that measured yield in the full-N treatment can serve as a proxy for Yp for each environment (i.e., Yp with no N limitation). In contrast, the measured zero-N yields in production environments exhibiting a Yp of >3.5 Mg ha^{-1} fell below the 1:1 line, and the magnitude of this yield difference between fertilized and non-fertilized treatments increased as the Yp of the production environment increased.

A statistically significant effect of N treatment on seed yield ($P<0.001$) was observed in the ANOVA (Table 2-2). When averaged over all environments, seed yield in the full-N treatment was 0.46 Mg ha^{-1} greater (i.e., 11% higher) compared to zero-N treatment (Figure 2-3). The ANOVA also revealed a significant environment x N interaction on seed yield ($P=0.025$), which was consistent with the significant upward departure of the observed linear regression slope of 1.2 from the null hypothesis of unity ($P<0.001$, Figure 2-3). For each unit Mg ha^{-1} increase in zero-N seed yield above a threshold of Yp of ca. 2.5 Mg ha^{-1}, there was a corresponding 1.2 Mg ha^{-1} increase in full-N seed yield. The yield difference between the full-N and zero-N treatments, increased with increasing Yp of the production environments, from near zero in low-Yp environments (2-3 Mg ha^{-1}) up to ca. 0.90 Mg ha^{-1} in production environments with a high-Yp of ca. 6 Mg ha^{-1} (Figure 2-3, inset).

Measured N uptake in zero-N maize plots located adjacent to the experiments indicated that indigenous N supply (excluding N fixation) did not vary greatly across environments, ranging from 133 to 148 kg N ha^{-1} across experiments in BA and from 98 to 150 kg N ha^{-1} in NE producer fields, except for Atkinson (196 kg N ha^{-1}). Hence, our comparison of N treatments along the range of low- to high-Yp environments was likely not confounded with co-variation in indigenous N supply.

Table 2-2. Analysis of variance for the effect of nitrogen (N) fertilizer on seed yield, seed number, and seed weight, aboveground dry matter at physiological maturity (ADM) and harvest index (HI), seed protein and oil concentration, and yields of those two seed constituents. Each variety x sowing date x year combination (Balcarce) or producer field (Nebraska) was considered to be a separate environment (E) relative to the N x E interaction in this analysis. Rep: replicate.

		Seed yield		Seed number		Seed weight		ADM		HI	
Fixed effects	d.f.	F	P > F	F	P > F	F	P > F	F	P > F	F	P > F
$E^{\dagger}$	24	32	<0.001	34	<0.001	27	<0.001	19	<0.001	4	<0.001
N	1	87	<0.001	27	<0.001	117	<0.001	33	<0.001	1	0.411
N x E	24	2	0.025	1	0.507	2	0.005	2	0.070	1	0.323
Random effects	d.f.	MS		MS		MS		MS		MS	
Rep (E)	75	330428		67219		77		2266459		0.003	
N x Rep (E)	75	123244		30753		24		1339567		0.002	
		Protein %		Oil %		Protein yield		Oil yield			
Fixed effects	d.f.	F	P > F	F	P > F	F	P > F	F	P > F		
$E^{\dagger}$	24	16	<.001	56	<.001	31	<.001	42	<.001		
N	1	275	<.001	41	<.001	159	<.001	63	<.001		
N x E	24	2	0.007	2	0.020	2	0.017	2	0.006		
Random effects	d.f.	MS		MS		MS		MS			
Rep (E)	75	0.940		0.249		38850		11536			
N x Rep (E)	75	0.419		0.106		15261		4478			

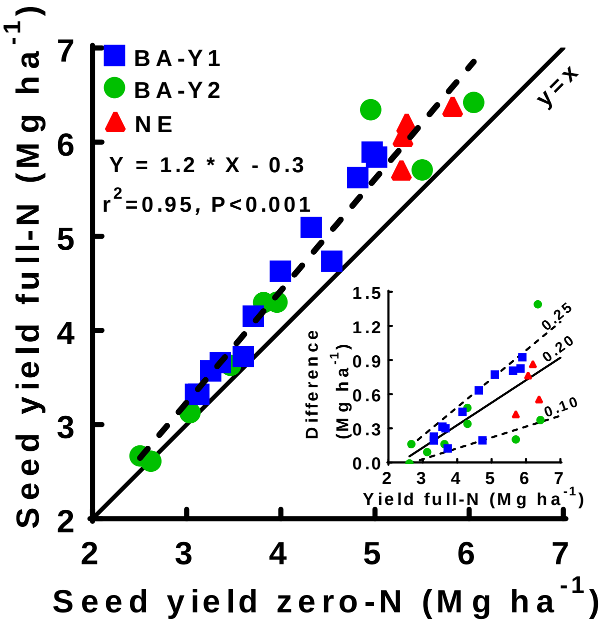

Figure 2-3. Seed yield in the full-N versus zero-N treatments. Each data point represents the average yield for a given year x sowing date x variety (Balcarce; BA) or producer field (Nebraska; NE). Experiments were conducted in BA over two crop seasons (BA-Y1 and BA-Y2) and at four sites in NE during one crop season. Solid diagonal line indicates y = x. Parameters of the fitted linear regression (dashed line) and coefficient of determination (r^2) are also shown. Inset shows the seed yield difference between the full-N and zero-N treatments relative to the full-N treatment yield. Also shown is the slope of the fitted linear regression (solid line; y = 0.2x – 0.46) and the slopes representative of the boundary functions for the 10th and 90th quantiles (dashed lines).

2.3.2. Drivers of seed yield differences between N treatments

Changes in both seed number and individual seed mass (i.e., weight) were consistent with the observed differences in seed yield between N treatments (Table 2-2, Figure 2-4). Averaged over all environments, the full-N treatment resulted in more seeds produced per unit of harvested area (2534 versus 2406 seeds m^{-2}) and also resulted in greater mass per seed (183 versus 175 mg seed^{-1}). In contrast to seed number, the seed weight trend line resided above the 1-to-1 line over the entire range of seed weight, suggesting that seed weight was persistently greater in full-N versus zero-N treatments. Interestingly, though N fertilization influenced both seed number and seed weight, a significant N x E interaction was detected only for the latter (Table 2-2). Similarly, N treatments accounted for a much larger portion of the observed variation in seed weight in relation with seed number (F-value: 117 versus 27, respectively).

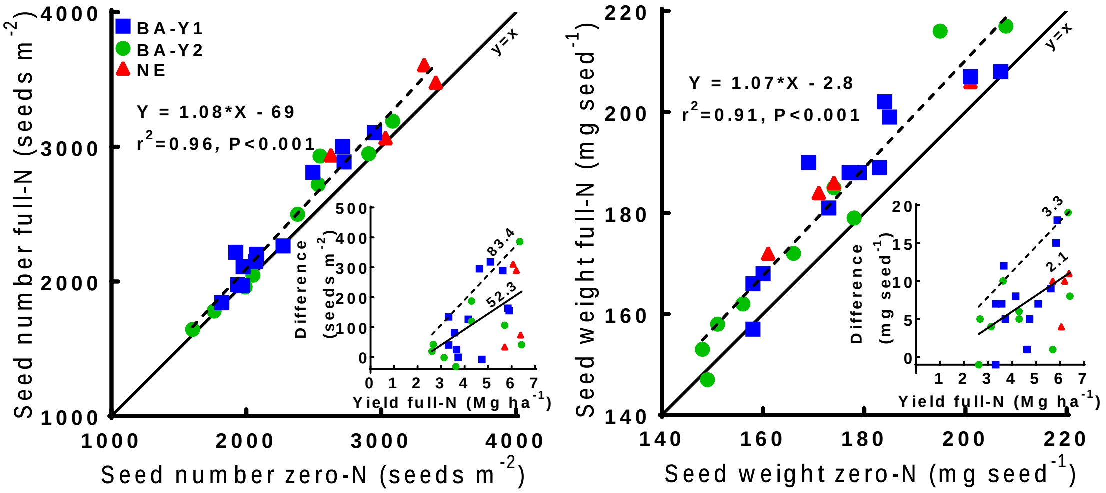

Figure 2-4. Seed number per unit harvested area (left) and individual seed mass (right) in full-N versus zero-N treatments. Each data point represents the average yield for a given year x sowing date x variety (Balcarce; BA) or producer field (Nebraska; NE).

Experiments were conducted in BA over two crop seasons (BA-Y1 and BA-Y2) and at four sites in NE during one crop season. Solid diagonal line indicates y = x. Parameters of the fitted linear regression (dashed line) and coefficient of determination (r^2) are also shown. Insets show the difference in seed number and weight between the full-N and zero-N treatments relative to the full-N treatment for those two measured variables. Also shown are the slopes of the fitted linear regressions (solid lines) and the slopes of the boundary function for the 90th quantile (dashed line). The boundary function for the 10th quantile is not shown due to the non-significance of that slope from zero.

Greater ADM was evident in the full-N versus zero-N treatments (Table 2-2, Figure 2-5). Across experiments, the full-N treatment generated an ADM that was 0.93 Mg ha^{-1} (9%) higher than that in the zero-N treatment. Notably, the ADM values measured in some of the high-yield environments in our experiment reached ca. 15 Mg ha^{-1}, and thus are close to the highest values reported to date for field-grown soybean in the literature (e.g., Setiyono et al., 2010; Van Roekel and Purcell, 2014). In contrast, sign and magnitude of differences in HI between full-N and zero-N treatments were not consistent across experiments, especially during the first crop season in Balcarce (Figure 2-5). Measured HI in the full-N treatments was slightly smaller (0.33 versus 0.34) and higher (0.39 versus 0.37) than zero-N treatments in environments below 3.5 Mg ha^{-1} or above 5 Mg ha^{-1} of Yp, respectively. Hence, changes in biomass partitioning along the range of Yp may explain why the consistent difference in ADM between full-N and zero-N treatments

(Figure 2-5) did not translate in an equally consistent seed yield response across the entire range of Yp (Figure 2-3).

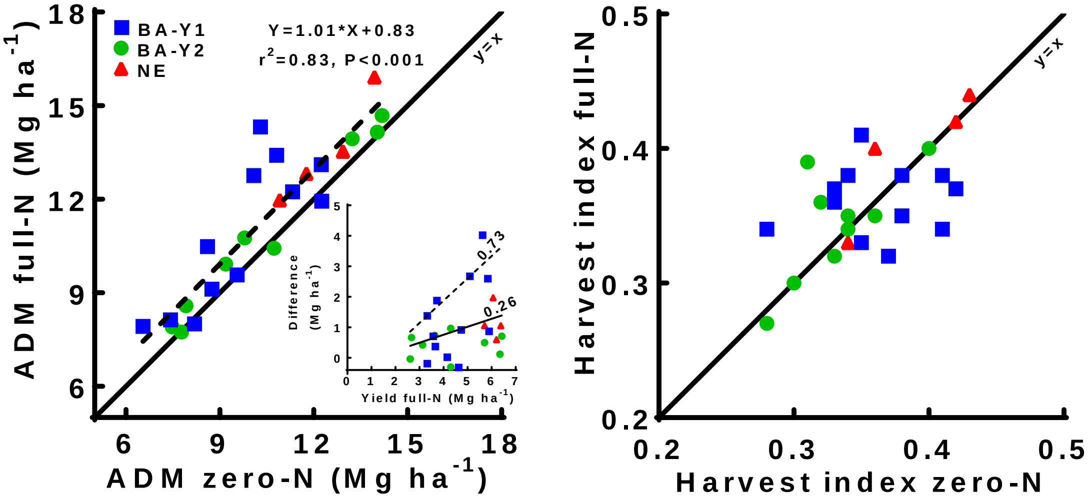

Figure 2-5. Aboveground dry matter (ADM) at physiological maturity (left) and harvest index (right) in full-N versus zero-N treatments. Each data point represents the average yield for a given year x sowing date x variety (Balcarce; BA) or producer field (Nebraska; NE). Experiments were conducted in BA over two crop seasons (BA-Y1 and BA-Y2) and at four sites in NE during one crop season. Solid diagonal line indicates y = x. Parameters of the fitted linear regression (dashed line) and coefficient of determination (r^2) are shown when significant. Inset show the difference in ADM and HI between the full-N and zero-N treatments relative to full-N treatment for those two measured variables. Slopes of the fitted linear regression (solid line) and the boundary function for the 90th quantile (dashed line) are shown only for ADM. The boundary function for the 10th quantile is not shown due to the non-significance of that slope from zero.

2.3.3. Soybean protein and oil as affected by N treatments

Averaged over all environments, the full-N treatment resulted in significantly greater yields of protein and oil ($P<0.001$) (Table 2-2, Figure 2-6). The protein and oil yield advantage arising from N fertilization averaged a respective 0.22 Mg ha^{-1} (15%) and 0.07 Mg ha^{-1} (10%). The steepness of the significant regression coefficients for the protein yield trend line (1.24) and oil yield trend line (1.18) relative to the null hypothesis of 1.0 (unity) is of interest. Clearly, when these two key soybean constituents were measured in units of dry matter harvestable per ha, N fertilization increased the (Mg ha^{-1}) values when going from low to high Yp environments (Figure 2-6). There was a small (0.3%), though statistically significant ($P<0.001$, Table 2-2) decrease in seed oil concentration arising from N fertilization when averaged over all environments (i.e., 20.5 versus 20.8 % for full-N and zero-N, respectively) (Figure 2-6, inset). However, that small decrease in percentage oil concentration was substantively offset in the oil yield calculation because of the 11% increase in seed yield generated by N fertilization. Seed protein concentration was significantly increased by N fertilization ($P<0.001$, Table 2-2). The increase, when averaged over all environments, was about 1.5 percentage points (i.e., 41.4 versus and 39.9% for full-N and zero-N, respectively) (Figure 2-6, inset). In contrast to oil yield, the large protein yield increase in the full-N treatment was attributable to N-fertilizer induced increases in both seed yield (11%) and protein concentration (4%).

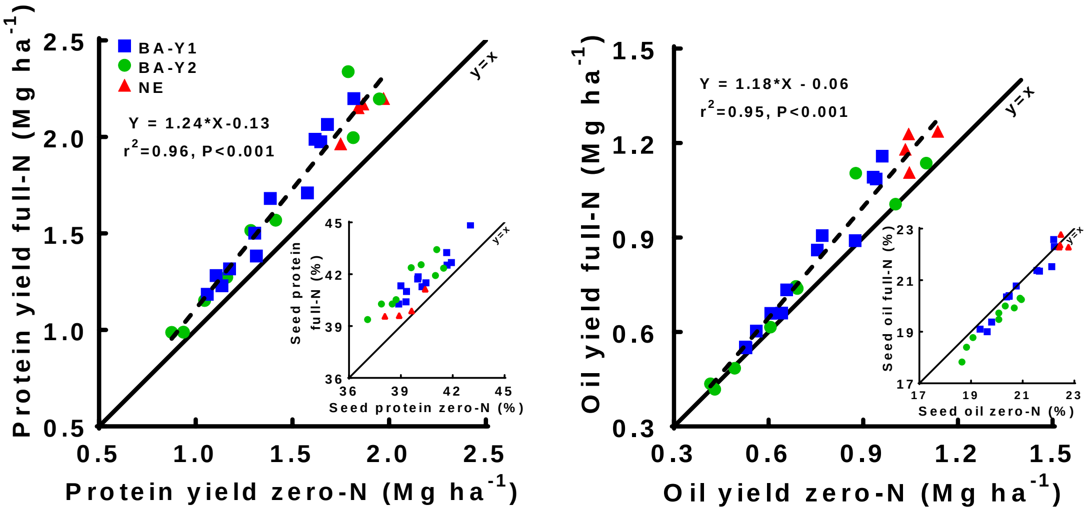

Figure 2-6. Seed protein yield (left) and seed oil yield (right) in full-N versus zero-N treatments. Each data point represents the average yield for a given year x sowing date x variety (Balcarce; BA) or producer field (Nebraska; NE). Experiments were conducted in BA over two crop seasons (BA-Y1 and BA-Y2) and at four sites in NE during one crop season. Solid diagonal line indicates y = x. Parameters of the fitted linear regression (dashed line) and coefficient of determination (r^2) are also shown. Insets show seed protein and seed oil concentration percentages relative to the zero-N treatments for those two measured variables.

Soybean seed processors who purchase soybeans are not interested in protein or oil yields. Instead, their primary interest is the percentages of protein and oil in the seed, and they will selectively purchase soybeans from location-year sites with higher than average percentages in one or both constituents. In our experiment, the relationship between protein yield and seed yield was curvilinear, suggesting a ‘dilution’ in seed protein was occurring across the wide range of increasingly greater zero-N treatment seed yields

(Figure 2-7, left). Protein concentration decreased (from ca. 41 to 38%) as the yield level in the zero-N treatment increased (from ca. 2.5 to 6 Mg ha^{-1}). In contrast, full-N crops maintained the same protein concentration across the wide range of measured full-N treatment seed yields (Figure 2-7, left). In other words, these observed patterns indicate that, when N fertilizer was used to enhance seed yield, the seed protein concentration at higher yield levels did not decline. The impact of N fertilization on seed oil was somewhat different in that the relationship of seed oil yield to seed yield was upwardly curvilinear for both the zero-N and full-N treatments (Figure 2-7, right).

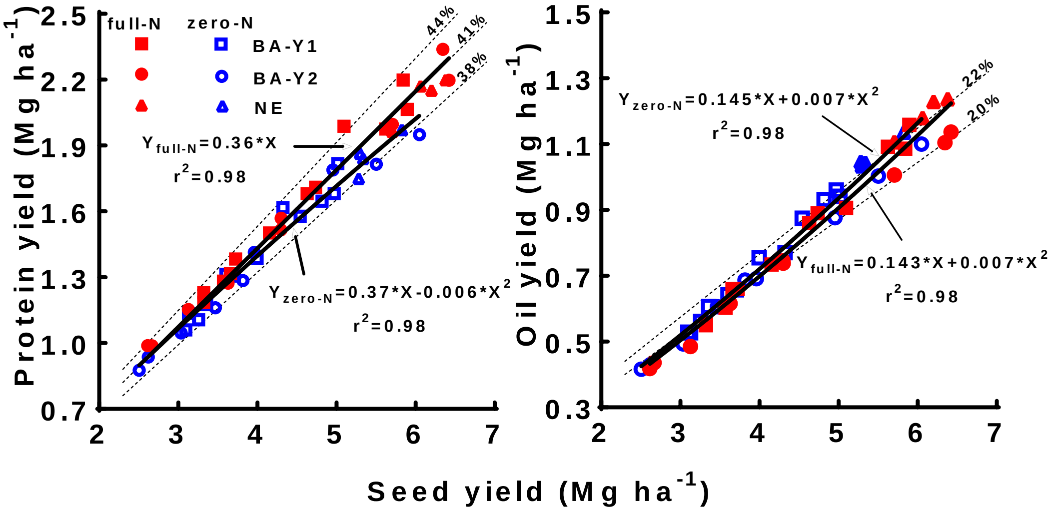

Figure 2-7. Protein (left) and oil (right) yields versus seed yield in full-N (solid red symbols) and zero-N treatments (empty blue symbols). Each data point corresponds to a sowing date x variety x N treatment (Balcarce; BA) or producer field x N treatment (Nebraska; NE). Experiments were conducted in BA over two crop seasons (BA-Y1 and BA-Y2) and at four sites in NE during one crop season. Fitted linear or quadratic equations (solid lines) were forced through zero; their equations are shown. Shown for

comparison are isolines for larger and smaller seed protein and oil concentration values (dotted lines).

2.4. Discussion

Findings from the present study challenge the hypothesis that N fixation can fulfill plant N requirements irrespective of the Yp of the production environment. Here we developed a seasonal N-fertilization protocol to ensure non-N limiting growing conditions for field-grown soybean. The protocol consisted on estimating total N crop requirements based on simulated site-specific Yp levels, while ensuring a timely synchronization between N demand and crop phenology by scheduling of the fertilizer applications in fractional time-step amounts to match seasonal pattern in crop N uptake dynamics. The protocol was implemented at sites with a wide range of Yp, ranging from ca. 2.5 to 6.5 Mg ha^{-1}, to evaluate the degree to which soybean yields may have been limited by N supply at those sites. We found a consistent increase in seed, protein, and oil yields in the full-N versus zero-N treatments, especially in environments with high Yp, without trade-offs on seed protein and oil concentration. Increases in primary yield components of seed number and individual seed mass (i.e., weight) were consistent in terms of accounting for the higher seed yield in the full-N treatment. In that regard, greater seed yield was also highly associated with higher ADM, while no consistent trend was found in biomass partitioning to seed (i.e., HI). We do not exclude the possibility that the yield difference found between zero and full-N treatments could also be partly related with smaller C cost due to reduced symbiotic N fixation in the full-N treatment (Ryle et al., 1979; Pate and Layzell, 1990; Connor et al., 2011). However, we note that (i) protein yields were higher in full-N versus zero-N treatments, suggesting greater N uptake in the full-N crops, and (ii) magnitude of yield increase was dependent upon the Yp of the production environment. These two observations were consistent with our hypothesis that there is a gap between crop N requirements and N supply (which is comprised of N fixation and soil mineralization) and that gap becomes detectably larger with the increasing N demand needed to support a higher Yp.

While we acknowledge that the N fertilizer amounts applied in the present study were far from being economically profitable (and environmentally sound), our N fertilizer protocol was specifically designed to allow us to experimentally identify a Yp threshold above which in situ N fixation and soil mineralization were not capable of meeting the crop N demand. On average, seed yield in the full-N treatment increased, relative to the zero-N treatment, by ca. 200 kg per Mg increase in Yp, but this N fertilizer induced yield response was experimentally evident only in crops with Yp level above 2.5 Mg ha^{-1}, suggesting that yield response to N fertilizer is more likely in fields with a Yp above this threshold. Our finding is not consistent with Ray et al. (2006) who found a persistent yield response to N fertilizer over a wide yield range, from 1.5 to 5.0 Mg ha^{-1}. We note, however, that most of the experiments in the lower yield range reported in the Ray et al. (2006) study were conducted in rainfed crop settings, for which N fertilizer may have helped to alleviate the effect of N limitation due to seasonally sporadic, temporary water deficits (Purcell, 2014). Likewise, yield responses to small N additions have been reported for low-yield environments with very low indigenous N supply, where a relatively small fertilizer N addition early in the season helps establish N fixation (Van Kessel and Hartley, 2000; Giller and Cadisch, 1995). In contrast, our experiments were conducted in fertile soils with relatively high indigenous N supply and in which irrigation was efficiently used to ensure that only non-water limiting conditions prevailed relative to our experimental objectives. Finally, our study set an upper limit with respect to soybean yield increases that could be induced by N fertilization. The upper limit for seed yield response to N fertilizer increased by 250 kg per Mg increase in Yp between the 2.5 Mg ha^{-1} Yp threshold and Yp levels approaching 6 Mg ha^{-1}. The maximum yield response we report here (≈ 0.9 Mg ha^{-1} at Yp=6 Mg ha^{-1}) is consistent with the upper range of soybean yield responses to N fertilizer reported by Salvagiotti et al. (2008) and Wilson et al. (2014).

Results presented in this paper challenge current estimates of Yp in soybean, and more broadly, legume crops. Findings reported here indicate that thoughtful focus on N supply during the temporal phases of seasonal crop development can help ensure non-N limited conditions in field-grown soybean. Hence, we argue that current estimates of Yp derived from field observations have likely underestimated soybean Yp (if N was limiting), given the lack of studies in which high-yield soybean has been grown following an explicit protocol to ensure ample N supply in time and space. The protocol developed in this study provided an experimental approach to minimize N limitation in high-yield conditions and thus ensured that measured yields can be taken as a reliable estimate of non-N-limited Yp. In our experiment, we found that crop model simulated Yp values closely matched full-N yields, indicating that crop simulation models are still robust at simulating Yp for soybean when N is not limiting. A possible explanation is that the procedure used to calibrate soybean models considers optimal leaf N for the different phenological stages, without limitations by soil nutrient availability or symbiotic N fixation in root nodules (Boote, et al., 2008; Setiyono et al., 2010 and references cited therein).

Finally, our results suggest that N fixation alone is not sufficient to fulfill soybean N demand in production environments with high Yp, which include irrigated soybean fields located in the central US Great Plains (Grassini et al., 2015). Hence, insufficient N supply may explain part of the current yield gap between simulated Yp and average producer yield reported for these cropping systems. For example, using Figure 2-3 as basis to determine the yield loss due to insufficient N supply for irrigated soybean in the US central Great Plains, given respective Yp and actual average producer irrigated yield of 5.7 and 4.5 Mg ha^{-1} (Grassini et al., 2015; USDA-NASS, 2015-2016; https://www.nass.usda.gov/Quick_Stats/), we inferred that at least half of the current estimated yield gap may result from a seasonal N supply limitation. In a broader context, we speculate that N supply will likely become (if not already) a major yield-limiting factor in soybean production systems with high Yp as producer yields in those systems continue to fine-tune their agronomic practices and adopt higher yielding cultivars (Grassini et al., 2014; Specht et al., 2014).

2.5. Conclusions

In the present study, we developed a novel N fertilization protocol to ensure non-N limiting conditions in field-grown soybean. The protocol was implemented across a wide range of environments of varying Yp to evaluate the degree of N limitation on seed yield and quality. Measured yield in treatments receiving N fertilizer closely matched simulated Yp. We found that soybean yields are limited by N supply in environments with Yp above ≈ 2.5 Mg ha^{-1}. Above this threshold, yield response to N fertilizer had an upper limit of 250 kg per Mg increase in Yp. Differences in seed yield between full-N and zero-N treatments were associated with higher seed number and weight and higher ADM. Protein and oil yields were also higher in the full-N treatments due to higher yields, with a slight increase and decrease in protein and oil concentration, respectively. Remarkably, full-N crops maintained the same seed protein concentration across the entire yield range and exhibited higher protein yields, especially at high yield levels. This study provides a framework to assess N limitations in field-grown soybean, and possibly other legume crops, and to explore opportunities to tune current N management in high-yield soybean cropping systems.

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CHAPTER 3: NITROGEN LIMITATION IN HIGH-YIELD SOYBEAN: SEED YIELD, N ACCUMULATION, AND N-USE EFFICIENCY

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Abstract

Recent reviews about soybean nitrogen (N) requirement have speculated about a possible N limitation in high-yield environments (>4.5 Mg ha^{-1}). However, these studies did not provide definitive experimental data to test that hypothesis and results from the literature are conflicting. To fill this knowledge gap, we evaluated N limitation across 13 high-yield soybean environments in Argentina and USA. Each experiment included a 'zero-N' treatment, which forced the crop to rely on biological N_2 fixation and indigenous soil N, and a 'full-N' treatment, which provided an ample fertilizer N supply during the entire crop cycle based on site-specific yield potential. Accumulated N in aboveground dry matter (ADM) measured in a N-omission maize plot grown adjacent to the soybean experiments was used to determine indigenous soil N supply. Soybean seed yield, protein and oil concentration, ADM, harvest index (HI), accumulated N in ADM, N harvest index (NHI), and seed N were measured in both treatments at physiological maturity. A simple conceptual framework relating ADM with accumulated N was used to assess treatment differences in ADM, accumulated N, and N-use efficiency (NUE). To account for treatment differences in seed biomass composition, mass-based ADM was expressed in glucose equivalents (ADM_e). Seed yield ranged from 4.6 to 6.7 Mg ha^{-1} (full-N) and 4.0 to 5.8 Mg ha^{-1} (zero-N), with the full-N treatment averaging 12% (0.6 Mg ha^{-1}) and 2.6% (9 g kg^{-1}) higher seed yield and protein concentration, respectively. The full-N treatment exhibited 18% (70 kg N ha^{-1}) and 14% (1.6 Mg ha^{-1}) greater accumulated N and ADM, respectively, compared with the zero N treatment, without changes in HI and NHI, but slightly lower NUE (29 versus 30 kg ADM kg^{-1} N in full and zero-N, respectively). However, NUE differences between treatments became indistinguishable when ADM was expressed as ADM_e (45 kg glucose kg^{-1} N). The (full-N minus zero-N) seed yield difference and the indigenous soil N supply were negatively associated. This research documented (i) the existence of N limitation in high-yield soybean, (ii) that seed yield and protein concentration increases were physiologically associated with changes in accumulated N (but not in NUE and/or NHI), and (iii) that the degree of N limitation is modulated by indigenous soil N supply. Findings from this study can serve as a basis to identify environments with the largest N limitation and thus guide N management in soybean.

Keywords: Glycine Max L.; soybean; yield; nitrogen; indigenous soil nitrogen

3.1. Introduction

Soybean [Glycine max (L.) Merr.] is an important legume crop because it is the predominant source of vegetable protein for food and feed in the world (Wilson et al., 2008). Soybean has a large nitrogen (N) requirement, accumulating ca. 80 kg N ha^{-1} in the aboveground dry matter (ADM) at the R7 stage[1] for each Mg of seed yield produced (Salvagiotti et al., 2008; Tamagno et al., 2017). Hence, a soybean crop that produces 4.5 Mg ha^{-1}, which is equivalent to current average irrigated US soybean yield, has an associated N requirement of ca. 360 kg N ha^{-1}. To fulfill this N requirement, soybean relies on biological N_2 fixation and other sources of N including soil N supply from organic matter (SOC) mineralization, dry and wet atmospheric deposition, N from irrigation water, and a small amount of N fertilizer applied as 'starter' at sowing by some producers (hereafter collectively termed 'indigenous soil N supply'). The negative association (trade-off) between N_2 fixation and indigenous soil N supply has been well-documented in the literature (Streeter, 1985; Salvagiotti et al., 2008, 2009; Santachiara et al., 2017; Tamagno et al., 2018). As soybean yield continues to increase due to breeding and agronomic management (Specht et al., 2014; Grassini et al., 2015), the question

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[1] In this report, we used the phenological stages defined by Fehr and Caviness (1977). V2: fully developed trifoliolate leaf at node above the unifoliolate node; V4: four nodes on the main steam with fully developed leaves beginning with the unifoliolate node; R1: (beginning of bloom) one open flower at any node on the main stem; R3: (beginning of pod setting) pod of 5 mm long at one of the four uppermost nodes on the main stem with a fully developed leaf; R5: (beginning of seed filling) a seed 3 mm long in a pod at one of the four uppermost nodes on the main stem with a fully developed leaf; R7: physiological maturity.

arises as to whether biological N_2 fixation and indigenous soil N supply will be sufficient to meet the crop N requirements in future high-yielding production scenarios.

Most recent reviews about the N requirement in soybean have speculated about a possible N limitation in high-yield soybean, sometimes referred as the ‘N gap’ (Salvagiotti et al., 2008; Ciampitti and Salvagiotti, 2018). However, the cited studies provided no definitive experimental data to test that hypothesis. Acquiring such data would require a side-by-side comparison of seed yield and N accumulated in ADM between a ‘control’ treatment (hereafter called ‘zero-N’) that forces the crop to rely on site-specific biological N_2 fixation and indigenous soil N supply, and a (‘full-N’) treatment specially designed to provide the crop with an ample N supply to optimally match seasonal crop N demand. The full N treatment would be expected to preclude the occurrence of any N limitation because any site-specific insufficient indigenous soil N supply is supplemented with added N. Likewise, it would neutralize the trade-off involving an expected depression in crop N acquired from N_2 fixation versus an expected elevation in crop N acquired from a greater soil N supply. This two-treatment comparison should be conducted in the absence of crop water and biotic stresses, so that any N limitation is not confounded with, or masked by, these stress factors. Additionally, to ensure a balanced comparison of the two treatments, a mass-based evaluation of treatment difference in biomass or resource-use efficiency must be accompanied by an energy-based, glucose equivalency evaluation (Penning de Vries et al., 1983; Amthor, 2010). This evaluation is crucial if the two contrasting N treatments generate differences in the concentrations of oil, protein, and carbohydrate constituents in seed biomass. Finally, to facilitate interpretation of experimental results across site-years, it would be relevant to have a measure of the site indigenous soil N supply, as this variable will likely influence the degree of N limitation and, therefore, differences in seed yield between the treatments. A robust evaluation of N limitation in high-yield soybean following these criteria has not yet been performed.

Previous studies have not investigated the drivers for the apparent N limitation in high-yield soybean in terms of changes in accumulated N, N harvest index (NHI) and/or physiological N-use efficiency (NUE; expressed as kg of ADM or glucose equivalents per kg accumulated N), and results from these previous studies are conflicting. In a recent study, Cafaro La Menza et al. (2017) compared soybean seed yields between a treatment wherein the crop had to rely on indigenous soil N supply and biological N_2 fixation (‘zero-N’), and a treatment in which the crop had access to an ample N supply during the entire crop season (‘full-N’). This experiment involved a wide range of irrigated production environments which, in the absence of water stress, generated seed yields ranging from 2.5 to 6.0 Mg ha^{-1} in the zero-N treatment. On average, seed yield was greater (11%) in full versus zero-N treatment; however, the magnitude of seed yield increase depended upon the relative productivity of the environment. Larger yield differences between treatments were observed in environments with a higher yield potential. Interestingly, seed protein concentration also increased in the full-N treatment. However, that study did not establish whether changes in seed yield and protein concentration between treatments were associated with changes in accumulated N, NUE, and NHI, or any combination of these variables. In a later study following a similar experimental protocol, Santachiara et al. (2018) found an increase in accumulated N (7%) in a full-N versus zero-N treatment in experiments conducted in rainfed production environments where seed yield ranged from 4.7 to 5.5 Mg ha^{-1} in the zero-N treatment. In contrast to Cafaro La Menza et al., (2017), there was no statistically significant difference in seed yield between the two treatments and seed protein was slightly lower (2%) in the full-N treatment as it can be inferred from their data.

The present study extends the previous assessment performed by Cafaro La Menza et al. (2017) by investigating the physiological drivers for seed yield and protein increase as a result of greater N supply, and evaluating how yield increases were modulated by the indigenous soil N supply. Here we evaluated seed yield and protein concentration, ADM, accumulated N, seed N, NHI, and NUE in irrigated experiments conducted in Argentina and USA that were explicitly managed to reach near-optimal growing conditions. Collectively, these two countries account for ca. 50% of soybean global production (FAOSTAT; http://www.fao.org/faostat/en/#data/QC). A zero-N treatment relying on indigenous soil N and biological N_2 fixation as N sources was compared to a full-N treatment designed to provide the crop with seasonally ample N supply. The objectives of the study were to (i) assess drivers for changes in seed yield and protein concentration between treatments in terms of accumulated N, NUE, and NHI, and (ii) evaluate the degree to which indigenous soil N supply modulated the yield difference between treatments.

3.2. Materials and methods

3.2.1. Experimental sites

Previous studies have used a yield threshold of 4.5 Mg ha^{-1} to identify high-yield soybean production environments where N supply from biological N_2 fixation and soil may not be sufficient to meet crop N requirements (Salvagiotti et al., 2008). In the present study, we used a subset of the experiments conducted in Nebraska, USA (NE) and Balcarce, Argentina (BA) reported in Cafaro La Menza et al. (2017). Additionally, we included four new experiments conducted in NE during 2017 (Table 3-1; Supplementary Table 3-S1). These experiments were explicitly selected to portray high-yield conditions. Simulated yield potential, based on site-specific weather and management practices (sowing date, maturity group and plant density) exceeded 4.5 Mg ha^{-1} in all these experiments (Cafaro La Menza et al., 2017) and measured yields ranged from 4.0 to 6.7 Mg ha^{-1}.

Experiments in NE were conducted in pivot-irrigated producer fields in 2016 and 2017 (total of seven field-year combinations). Experiments in BA were conducted in a factorial arrangement including different sowing dates and variety maturity groups during the 2014/2015 season (total of six sowing date-variety combinations). For simplicity, each field-year in NE or sowing date-variety combination in BA is referred to as an ‘environment’ (total: 13). Each environment included two N treatments (full-N and zero-N), with four replicates each (see Section 2.2). Experiments followed a completely randomized design in NE and a split-split plot design in BA. Crops were grown with the explicit goal of eliminating any yield-limiting and -reducing factor (except for N in the zero-N treatment). In all cases, seeds were treated with fungicide and insecticide but only inoculated in BA using the best available products in the market and following recommended inoculation practices (Nitragin Optimize II® Pack Apron® & Jumpstart). Seeds received no inoculant in the case of NE, which is the common producer practice for fields with a prior crop history of soybean in the US Corn Belt, given the lack of yield response to inoculation in high-yield environments (de Bruin et al., 2010; Leggett et al., 2017 and references cited therein). Detailed information about the experiments can be found in Cafaro La Menza et al. (2017).

A N-omission plot located adjacent (<30 m) to the soybean experiment in each NE or BA environment was sown with maize to estimate indigenous soil N supply by measuring the accumulated N in maize ADM at physiological maturity (Janssen et al., 1990; Setiyono et al., 2011). In each environment, the maize plot (9.1 x 11 m and 3.8 x 10 m in NE and BA, respectively) was sown on the same date as soybean and did not receive any N fertilizer (but received P and K fertilizer). In the case of BA, there were four maize plots per sowing date (one adjacent to each replicated main plot); in NE, there was only one plot per environment. Final count stand was 10 and 9 plants m^{-2} in BA and NE, respectively.

Plots received irrigation to avoid water limitation and were kept free of weeds, diseases, and pathogens during the entire crop season. Hybrids were selected to portray modern high-yield cultivars with broad adaptability and good pest resistance (DKC61-54RIB in NE and DK 692 VT3P in BA) and have similar growth duration to the soybean variety planted in each environment.

3.2.2. Nitrogen treatments and measurements

Two N treatments were compared in each environment: a 'zero-N' treatment that relied on indigenous soil N and biological N_2 fixation sources, and a 'full-N' treatment that received sequential applications of N fertilizer to ensure non-N limiting conditions. Total N fertilizer for the full-N treatment was calculated based on (i) site-specific yield potential, (ii) soybean N requirement per unit of seed yield (80 kg N Mg^{-1}; Salvagiotti et al., 2008; Tamagno et al., 2017), and (iii) an estimated fertilizer N recovery use efficiency ranging from 70 to 90% across environments to account for unavoidable N losses. The high recovery efficiency is justified given that the total N fertilizer rate was split into five applications, with timing and amount of each split adjusted to optimally match the expected crop N requirement. Total amount of N fertilizer ranged from 540 to 870 kg N ha^{-1} across environments and was applied as urea. Total N fertilizer was split into five applications (at V2, V4, R1, R3, and R5) in increasing amounts (10%, 10%, 20%, 30%, and 30% respectively) to mimic the seasonal pattern of N accumulation in soybean (Thies et al., 1995; Bender et al., 2015). Detailed information about the protocol followed in the full-N treatment can be found in Cafaro La Menza et al. (2017).

At physiological maturity (R7 stage), 1-m row of consecutive plants in each replicate, surrounded by two rows receiving same N treatment, was clipped at soil surface, and separated into five fractions: green leaves, senesced leaves, stems plus petioles, pod walls, and seeds. These five fractions, plus a composite of all abscised leaves collected on a weekly basis beginning at R3 (using mats placed between plant rows), were oven-dried at 70 °C until reaching constant weight. The weights of those six fractions were summed to calculate ADM at R7. Each ADM fraction (green, senesced, and abscised leaves, stems plus petioles, pod walls, and seeds) was ground separately using a Wiley mill (1-mm screen mesh). N concentration was determined using a dry combustion-based analyzer (LECO Corporation, St Joseph, MI). Accumulated N was calculated as the sum of N content in the six ADM fractions. Harvest index (HI) was calculated as the ratio between seed dry matter and ADM. Similarly, NHI was calculated as the ratio between seed N and accumulated N. Much larger areas of plant samples (1.8 and 4.6 m^2 in BA and NE, respectively) were collected from the two center rows in each plot and subsequently threshed to obtain an end-of-season estimate of seed yield. Soybean seed carbohydrate, protein, and oil concentrations (in units of g constituent kg^{-1} seed) were determined using near-infrared (NI) analysis (FOSS Infratec™ 1241). Seed yield and protein and oil concentration were adjusted to 0.130 kg H_2O kg^{-1} seed, while ADM, and HI were reported on an oven-dry moisture basis.

To determine maize ADM, grain yield, and accumulated N (the latter was used to estimate the indigenous soil N supply at each of the 13 experimental sites), the soybean sampling procedure was repeated for each maize plot except that: (i) one sample per plot was collected in BA, whereas three (sub-) samples were collected from the single maize plot in NE, and (ii) grain yield was expressed at 0.155 kg H_2O kg^{-1} seed. To determine the degree to which indigenous soil N supply could be successfully predicted from soil properties, soil samples were collected at sowing from the maize plots to determine soil texture (clay, silt, and sand fractions), N-NO_3^- concentration, and soil organic matter. In each environment, three or four soil cores were collected from the maize plot, split into 0-0.3, 0.3-0.6, 0.6-0.9, and 0.9-1.2 m segments, and combined into one composite sample per depth. In addition, total irrigation amount from sowing to soybean R7 stage was recorded for each environment using a flowmeter installed on each irrigation well. The ground water concentration of N-NO_3^- was determined from an irrigation water sample collected at each site at soybean crop stages R3 or R5, and then used to calculate the N contribution to the soil supply via irrigation water application.

Following Penning de Vries et al. (1983), soybean ADM (seed and non-seed biomass) was expressed as glucose equivalents (ADM_e) to account for possible differences in biomass constituent concentrations between treatments (method I). We assumed one kg of glucose to be equivalent to 0.33, 0.41, and 0.83 kg of lipid, protein, and carbohydrate. For seed biomass, we calculated glucose equivalents based on NI-measured lipid, protein, and carbohydrate concentration. In the case of non-seed aboveground biomass, protein concentration was estimated based on measured N content in each ADM fraction, using a 6.25 N-to-protein percentage conversion factor. Lipid fraction in non-seed biomass is expected to be very low and thus ignored (<15 g kg^{-1}; Peiretti et al., 2018). Regarding ADM mineral fraction for calculating ADM_e, we used an ash content of 6 and 5 % for seed and non-seed biomass, respectively. These ash percentages were determined by combustion (500°C for 4 h) of a subset of 96 samples. We cross-validated estimates of ADM_e using an independent methodology (method II) based on heat of combustion, organic N, and ash concentration (Williams et al., 1987; Amthor et al. 1994). Heat of combustion was determined for each ADM fraction (green, senesced, and abscised leaves, stems plus petioles, pod walls, and seeds) using a Parr® 1108 oxygen bomb calorimeter. The cross-validation was performed based only on the samples collected in NE (seven environments).

3.2.3. Conceptual framework for investigating N limitation in soybean

Drivers for differences in seed yield between treatments were assessed using a simple framework relating ADM with accumulated N (Fig. 1a). We used ADM instead of seed yield because the two N treatments did not significantly differ in HI and NHI. The slope of the relationship between ADM and accumulated N represents the NUE. Plausible changes in NUE, ADM, and/or accumulated N could be hypothesized when going from a zero-N (reference) scenario (box-1 in Fig. 1b) to a full-N scenario (box-2, -3, -4, -5 in Fig. 1b). If N_2 fixation plus indigenous soil N supply in the zero-N treatment is a limiting N scenario, and the full-N treatment provides an ample soil N supply to mitigate that N limitation, then one could envision two possibilities as to how that mitigation occurs: (i) ADM and accumulated N are higher, but with a reference NUE (i.e., going from 1 to 4), and (ii) same as (i), except that NUE is higher than the reference NUE (i.e., from 1 to 5). Previous studies have indicated that it may be the case of a larger carbon cost of N derived from N_2 fixation versus assimilating N absorbed from the soil (Andrews et al., 2009; Connor et al., 2011; Tamagno et al., 2018). This possibility is portrayed in the scenario in which ADM and NUE are higher but no change in accumulated N (i.e., going from 1 to 2). Likewise, some researchers have used the term “luxury consumption of N” to refer cases in which a higher accumulated N does not increase ADM, thereby resulting in a lower NUE, i.e., going from 1 to 3 in our scheme (Lemiare and Gastal, 2009; Santachiara et al., 2018). A separate analysis of NUE using ADM_e (instead of ADM) was performed to account for differences in seed biomass composition between treatments.

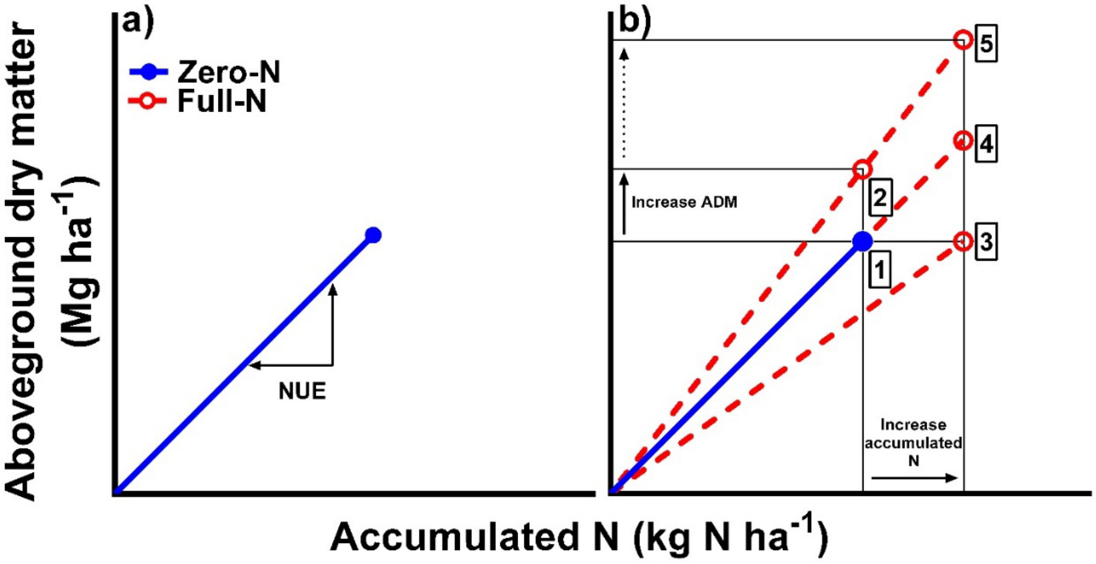

Figure 3-1. Schematic illustration of the linear response of ADM to accumulated N (both measured at R7), for which the slope is physiological nitrogen use efficiency (NUE). These three parameters provided a conceptual framework used in the present study to evaluate the nature of nitrogen limitation in high-yielding soybean systems. **(a)** zero-N treatment NUE slope (blue line & solid symbol); **(b)** relative to zero-N reference NUE (box 1), four plausible responses to the full-N treatment are shown (boxes 2, 3, 4, & 5) involving three differing NUE slopes (lower, same as reference, or higher) associated with an increase only in ADM (from 1 to 2), only in accumulated N (from 1 to 3), or both (from 1 to 4 or 5). See Section 2.3 for additional details.

3.2.4. Data analysis

A combined analysis of variance (ANOVA) was conducted to test the hypothesis that ample fertilizer N supply (full-N) treatment increases seed yield, seed protein and oil concentration, ADM, HI, accumulated N, seed N, and NHI (SAS® PROC MIXED v.9.3; Moore and Dixon, 2015). Experiments (in BA and NE) were not too different in relation with their variances ($F_{max} < 6$); hence, our combined ANOVA can be considered robust (Milliken and Johnson, 2009). In this analysis, each combination of year, sowing date, variety, and location was referred to as an environment. Each environment had two N treatments (full-N and zero- N), with four replicates. Environment and N treatments were treated as fixed effects. This analysis provided an estimate of the overall significance of the N treatment across environments. Although environments cannot be directly compared, the environment x N treatment interaction can be used to evaluate consistency of the N treatment response.

Linear regression analysis was used to investigate relationship between (i) ADM (or ADM_e) and accumulated N across treatments (hypotheses stated in Fig. 1b), and (ii) seed yield difference (full-N minus zero-N) and indigenous soil N supply to test whether increases in indigenous soil N supply decreases seed yield difference between treatments or not. After estimating the slope, intercept (if applicable), and coefficient of determination (r^2), F-tests were used to evaluate the statistical significance between treatments slopes, and intercepts difference relative to a null hypothesis of intercept equal to zero using GraphPad®. Regressions were forced through zero when the null hypothesis of zero intercept was not rejected, unless there was a biological rationale for a non-zero intercept. Data from separate N treatments were pooled together when regression analysis indicated no statistically significant difference between their intercepts and slopes (as determined based on F-tests). Pearson's correlation analysis was used to explore relationships between indigenous soil N supply, soil properties at sowing, and groundwater N delivered to the plots via irrigation and test whether any individual soil property or the groundwater N delivered can predict the amount of indigenous soil N supply or not.

3.3. Results

3.3.1. Influence of N treatment on seed yield, protein concentration, and N accumulation

Environment-specific seed yield ranged from 4.6 to 6.7 Mg ha^{-1} in the full-N treatment and from 4.0 to 5.8 Mg ha^{-1} in the zero-N treatment (Table 3-1 and Fig. 2a). Notably, accumulated N was higher in the full-N versus zero-N treatment across the 13 environments (Tables 1-2 and Fig. 2c). This finding provides empirical evidence of the existence of a N limitation that was overcome by supplementing the N supply from soil and N_2 fixation with N fertilizer in the full-N treatment. Mean seed protein (but not oil) concentration was also higher in the full-N versus zero-N (356 versus 347 g kg^{-1}), which was not unexpected, given the higher seed N (318 versus 273 kg ha^{-1}) in full-N (Tables 1-2 and Fig. 2d). Seed yield and accumulated N were, on average, 0.6 Mg ha^{-1} (12%) and 70 kg N ha^{-1} (18%) higher in full-N versus zero-N contrast. Though ADM averaged was 1.6 Mg ha^{-1} (14%) higher in the full-N versus zero-N comparison, the N treatment difference was essentially nil for HI or NHI (Tables 1-2, and Fig. 2b). When evaluated on a glucose-equivalent basis, the advantage of the full-N treatment was 13 and 15% greater for seed yield and ADM_e, respectively (Table 3-3), which was not much different than the 12 and 14% advantage on a mass basis (Table 3-2), with differences in HI being near zero in both cases.

Table 3-1. Mean seed yield, seed protein and oil concentration, aboveground dry matter (ADM), harvest index (HI), accumulated nitrogen (N) in ADM, seed N, and N harvest index (NHI) at physiological maturity (R7 stage) in the two N treatments in each of the 13 environments.

Location	Sowing date	MG	N treatment	Seed yield ($Mg\ ha^{-1}$)	Seed protein ($g\ kg^{-1}$)	Seed oil ($g\ kg^{-1}$)	ADM ($Mg\ ha^{-1}$)	HI	Accumulated N ($kg\ N\ ha^{-1}$)	Seed N ($kg\ N\ ha^{-1}$)	NHI
Balcarce	Nov 1, 2014	2.2	zero	5.0	362	196	10.8	0.41	387	265	0.71
			full	5.8	376	198	13.4	0.38	510	323	0.63
		3.8	zero	4.8	342	195	10.3	0.41	370	242	0.65
			full	5.6	351	194	14.3	0.34	490	298	0.61
		4.6	zero	5.0	338	198	12.2	0.35	411	252	0.62
			full	5.8	351	194	14.4	0.35	509	303	0.60
	Nov 27, 2014	2.2	zero	4.3	374	187	10.1	0.38	361	238	0.66
			full	5.1	390	186	12.8	0.35	476	307	0.65
		3.8	zero	4.5	348	189	11.3	0.35	380	231	0.61
			full	4.7	363	190	12.2	0.33	441	255	0.58
		4.6	zero	4.0	348	195	12.3	0.28	398	204	0.51
			full	4.6	364	195	11.9	0.34	422	254	0.60
Mead	May 8, 2016	2.7	zero	5.3	345	184	12.8	0.36	419	318	0.76
			full	6.2	347	188	13.7	0.39	460	367	0.80
	May 6, 2017	2.7	zero	5.4	349	178	11.3	0.41	370	303	0.82
			full	6.7	352	177	13.1	0.44	459	381	0.83
Saronville	April 26, 2016	2.4	zero	5.8	338	187	12.1	0.42	408	292	0.72
			full	6.4	345	186	13.4	0.41	436	345	0.79
	April 23, 2017	3.3	zero	5.7	332	192	13.1	0.38	401	302	0.75
			full	6.4	336	187	14.7	0.38	485	356	0.73
Smithfield	May 13, 2016	2.4	zero	5.3	331	193	10.4	0.44	364	291	0.80
			full	5.7	344	194	11.5	0.43	384	297	0.77
	May 25, 2017	2.7	zero	5.5	352	188	11.2	0.43	367	281	0.77
			full	5.5	349	186	12.4	0.39	432	322	0.74
Atkinson	April 24, 2017	2.7	zero	5.5	356	193	13.1	0.37	436	333	0.76
			full	5.7	359	197	13.3	0.38	466	321	0.68

Table 3-2. Analysis of variance for effect of nitrogen (N) treatment on seed yield, seed protein and oil concentration, aboveground dry matter (ADM), harvest index (HI), accumulated N in ADM, seed N, and N harvest index (NHI) at physiological maturity (R7 stage). Each of the 13 location × year x sowing date x variety maturity group combinations was considered to be a separate environment (E) for the N × E interaction in this analysis. Also shown are the F-test values and probabilities for the fixed effects, mean squares (MS) for the random effects, estimated means for each treatment, and a test of whether the treatment mean difference was significantly different from zero.

		Seed yield (Mg ha^{-1})	Seed protein (g kg^{-1})	Seed oil (g kg^{-1})	ADM (Mg ha^{-1})	HI	Accumulated N (kg ha^{-1})	Seed N (kg ha^{-1})	NHI
Fixed effects	d.f.	F	F	F	F	F	F	F	F
E	12	8.6***	38.9***	15.7***	2.9**	10.5***	2.3*	10.8***	29.1***
N	1	58.7***	77.7***	0.6	59.6***	0.7	104.5***	52.8***	3.0
N x E	12	1.31	3.3**	2.0*	2.5*	2.0*	2.6*	1.4	5.8***
Random effects		MS	MS	MS	MS	MS	MS	MS	MS
Rep (E)	39	0.30	34.9	15.1	2.0	0.001	2165	966	0.0020
N x Rep (E)	38	0.17	24.5	6.9	1.1	0.001	1239	899	0.0009
Estimated means									
Full-N		5.7	356	190	13.2	0.38	460	318	0.69
Zero-N		5.1	347	191	11.6	0.38	390	273	0.70
Difference (full-N -zero-N)		0.6***	9***	-1	1.6***	nil	70***	44***	-0.01

Asterisks indicate statistical significance at *p<0.05, **p<0.01, and ***p<0.001.

Table 3-3. Analysis of variance for effect of nitrogen (N) treatment on glucose-equivalent basis seed yield (SY_e), aboveground dry matter (ADM_e), and harvest index (HI_e) at physiological maturity (R7 stage). Each of the 13 location × year x sowing date x variety maturity group combinations was considered to be a separate environment (E) for the N × E interaction in this analysis. Also shown are the F-test values and probabilities for the fixed effects, mean squares (MS) for the random effects, estimated means for each treatment, and a test of whether the treatment mean difference was significantly different from zero.

		SY_e (Mg glucose ha^{-1})	ADM_e (Mg glucose ha^{-1})	HI_e
Fixed effects	d.f.	F	F	F
E	12	8.6***	5.8***	11.5***
N	1	65.2***	146.8***	1.1
N x E	12	1.2	4.4***	2.3
Random effects		MS	MS	MS
Rep (E)	39	0.94	5.1	0.002
N x Rep (E)	38	0.55	1.3	0.001
Estimated means				
full-N		10.2	21.1	0.49
zero-N		9.0	18.3	0.49
difference (full-N -zero-N)		1.2***	2.8***	-0.01

Asterisks indicate significance at *p<0.05, **p<0.01, and ***p<0.001.

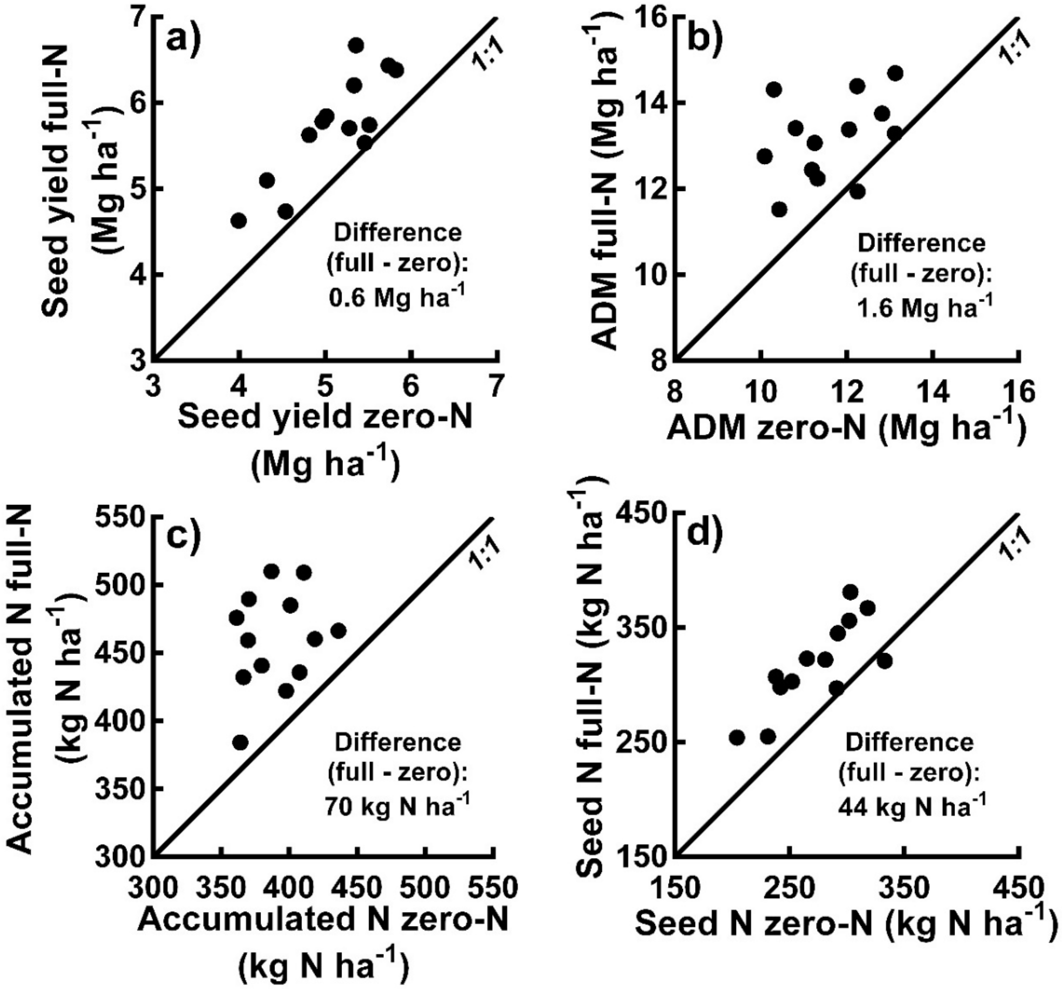

Figure 3-2. Scatter plot comparison of full-N (a) seed yield, (b) aboveground dry matter (ADM), (c) accumulated N, and (d) seed N relative to their respective zero-N counterpart values in each of the 13 environments. Difference values averaged over all environments are presented in each panel. Each of the 13 data points represent a location x crop season x sowing date x variety combination treated as an environment. The solid diagonal line in each plot indicates the 1:1 line. Differences were statistically significant from zero ($p<0.001$).

3.3.2. Influence of N treatment on accumulated N vs. N use-efficiency

Variation in ADM across environments and N treatments were clearly related to differences in accumulated N (Fig. 3a). As mentioned previously, N accumulation was higher in full-N versus zero-N treatment. However, the full-N treatment exhibited a slightly (4%) lower NUE slope compared with the zero-N treatment (29 versus 30 kg ADM kg^{-1} N; F-test p=0.04). The slightly lower NUE would, in our conceptual framework (Fig. 1b), be equivalent to the box-3 dashed NUE line. However, when ADM was expressed in glucose equivalents as ADM_e, the NUE for the full-N and zero-N treatments were not significantly different (F-test; p=0.14) (Fig. 3b). This finding justified pooling the two N treatment ADM_e data sets and then using a common linear regression to estimate NUE, which turned out to be 45 kg glucose kg^{-1} N. The regression based on the pooled ADM_e data had greater explanatory power compared with the regression analyses based on mass-basis ADM data ($r^2 = 0.87$ in Fig. 3b versus 0.74 and 0.60 in Fig. 3a). The ADM_e values generated with methods I and II (described in Section 2.2) were strongly associated (Fig. 3c), indicating that our estimates of ADM_e were robust. To summarize, differences in NUE between full and zero-N treatments became undistinguishable from zero when the mass-based NUE was converted to an energy-based NUE to account for differences in seed protein concentration between N treatments.

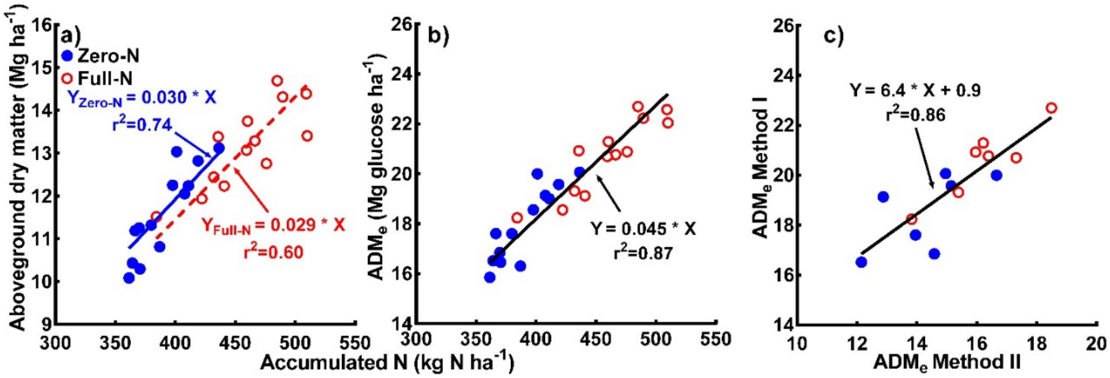

Figure 3-3. (a) Relationship between aboveground dry matter (ADM) and accumulated nitrogen (N) in the zero-N (solid blue symbols) and full-N treatments (empty red symbols). Separate linear regressions were fitted to the zero-N (solid line) and full-N treatments (dashed line). (b) Relationship between glucose-equivalent basis aboveground dry matter (ADM$_e$), and accumulated N. Each data point represents a location × year × variety x sowing date x variety combination (i.e., environment). (c) Relationship between two ADM$_e$ estimates: method I based on tabular data for oil, protein and carbohydrate construction costs reported by Penning de Vries et al. (1983), and method II based on estimates calculated from the measured heat of combustion and concentrations of N and ash following Williams et al. (1987).

3.3.3. Influence of indigenous soil N supply on N limitation

Magnitude of seed yield difference between treatments ranged from nil to 1.3 Mg ha^{-1} across the 13 environments (Table 3-1). Using the accumulated N measured in the N-omission maize plots, it was possible to estimate the amount of indigenous soil N supply in each environment, which ranged from 63 to 208 kg N ha^{-1} (Fig. 4a). Soybean yield difference (full-N minus zero-N) declined by ca. 6 kg ha^{-1} per kg ha^{-1} unit of increased indigenous soil N supply (Fig. 4a). This finding indicates that N limitation expressed as the yield difference between treatments was modulated by the amount of indigenous soil N supply of the production environment. No specific soil property (clay, silt, and sand contents, N-NO_3^- at sowing, and soil organic matter) or N from groundwater was closely associated with the maize-based predicted indigenous soil N supply (Pearson's r $\leq |0.68|$; $p \geq 0.06$; Supplementary Table 3-S2). In contrast, indigenous N supply was strongly correlated with the measured grain yield in the N-omission maize plot (Fig. 4b).

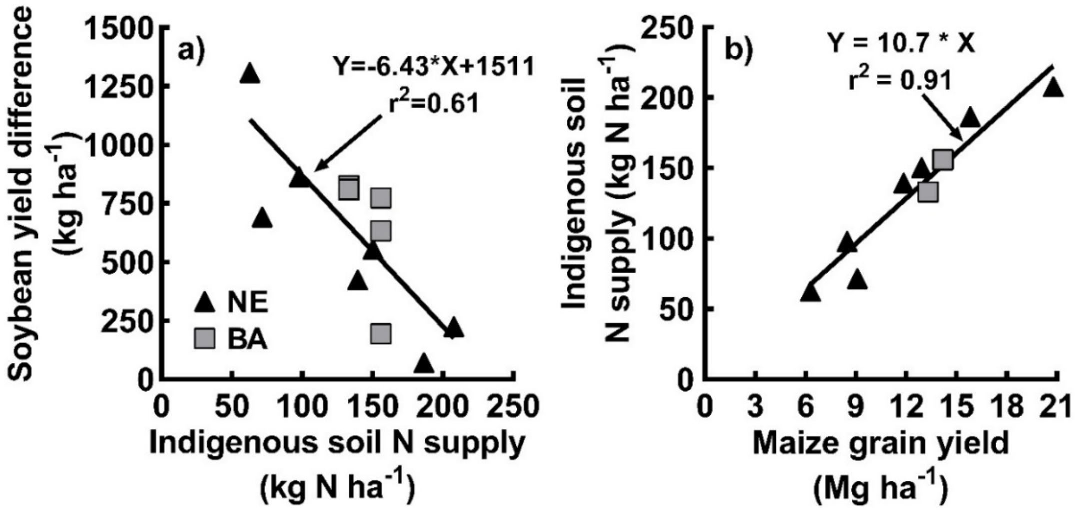

Figure 3-4. (a) Soybean seed yield difference between full-N and zero-N treatment versus indigenous soil nitrogen (N) supply. The latter was estimated from accumulated N in a N-omission maize plot located adjacent to the 13 soybean environments – seven in Nebraska (NE) and six in Balcarce, Argentina (BA). **(b)** Relationship between indigenous soil N supply and maize grain yield showing the predictive power of measured maize yield in an N-omission plot at estimating soil N supply. Note that not all expected data points are visible due to overlapping graph coordinate positions.

3.4. Discussion

In this study, differences in soybean seed yield and accumulated N between a zero-N treatment (in which the crop must rely on biological N_2 fixation and indigenous soil N supply) and a full-N treatment (in which the crop receives an ample N supply) were experimentally evaluated in 13 high-yield environments in USA and Argentina that were explicitly managed to eliminate other possible yield-reducing and -limiting factors. A seed yield and accumulated N threshold of 4.5 Mg ha^{-1} and 370 kg N ha^{-1}, respectively, have been suggested in the literature to define soybean production environments where N supply might become limiting (Salvagiotti et al., 2008; Ciampitti and Salvagiotti, 2018). In all our 13 environments, measured yields and accumulated N in the full-N treatment exceeded these thresholds, whereas for zero-N treatments, 11 (85%) and 10 (80%) environments did so for yield and accumulated N, respectively. Differences in seed yield and protein concentration between treatments were attributed to larger N accumulation in the full-N treatment, which, in turn, led to higher ADM and seed N, without detectable changes in HI and NHI. These results indicate that the zero-N treatment had an in-season N shortage, and consequently, accumulated N, ADM, seed yield and protein concentration were reduced in comparison to their non-N limiting counterpart values in the full-N treatment.

Our findings differed from those of Santachiara et al. (2018), who observed a slight increase in accumulated N and reduce NHI in the full versus zero-N treatment, but no detectable seed yield difference and reduced seed protein concentration (as inferred from their data) in the full-N treatment in rainfed soybean in central Argentina. Reported precipitation in Santachiara et al. (2018) suggested that water limitation during reproductive stages may have limited crop growth during critical phases of yield determination, thus masking the potential yield benefit derived from the extra N accumulation in the full-N treatment. On the other hand, our study extended the previous assessment conducted by Cafaro La Menza et al. (2017), who demonstrated significant seed yield and protein concentration increases in the full versus zero-N treatment, and indicated that these differences were attributable to higher accumulated N, thereby confirming previous speculations about the existence of a “N gap” in soybean (e.g., Salvagiotti et al., 2008; Ciampitti and Salvagiotti, 2018). The ample soil N supply, provided in the full-N treatment, led to a substantial percentage increase in seed protein content of +2.6% (i.e., 356/347 = 1.026) versus the zero-N treatment, but was surprisingly accompanied by a near-zero decrease in seed oil content of just -0.5% (190/191). This apparent decoupling of the well-known highly negative association of seed protein with oil seems to be an apparent reversal of frequent negative association of seed protein with yield (Chung et al., 2003) is an intriguing finding worthy of more research.

Our study also stressed the importance of accounting for N treatment-induced changes in the seed biomass constituents (oil, protein, and carbohydrate) influencing the comparison of NUE between treatments. For example, the higher accumulated N but a smaller NUE in the full-N versus zero-N treatment (using a mass-based ADM) would have suggested the occurrence of some degree of ‘luxury N consumption’ (Lemiare and Gastal, 2009). However, there was no difference in NUE when ADM was expressed in energy-based (ADM_e), indicating that the apparent ‘luxury N consumption’ detected on a mass-basis was really due to a higher seed protein concentration in the full-N treatment. We acknowledge a previous study suggesting the occurrence of N luxury consumption in soybean based on analysis of N dilution curves (e.g. Santachiara et al., 2018); however, this previous study assessed N dynamics only up to the R5 stage without considering the substantive N remobilization from vegetative organs to seeds during seed filling (Sinclair and de Wit, 1978; Salvagiotti et al., 2008; 2009). Our energy-based estimates of ADM_e in the 13 environments demonstrate that NUE in our study did not decrease or increase, despite the significant increases in both ADM and accumulated N (i.e., consistent with the box-1 to box-4 model shown in Fig. 1b). Thereby, we highlight the importance of calculating physiological efficiencies on an energetic-basis in addition to mass-basis especially for crop species with high oil and/or protein concentrations in their harvestable organs, as it is the case for soybean (Amthor et al., 2010).

In our study, the magnitude of seed yield difference between N treatments was modulated by indigenous soil N supply - yield difference was higher when the indigenous soil N supply was lower. We acknowledge that our estimates of indigenous soil N supply derived from accumulated N in N-omission maize plots might have been biased by temporal and spatial differences between maize and soybean root systems and N uptake (Giller, 2001). Though a soybean-based estimation of indigenous soil N supply would be desirable, it would require the use of isogenic nodulating and non-nodulating line pairs for a ‘modern’ soybean cultivar of high genetic yield potential in each of the major MG zones which, unfortunately, are not yet available. Still, we propose here that the strong relationship between full-N minus zero-N seed yield difference and maize-based indigenous soil N supply supports the measurement of the latter in future experiments to better understand the mechanistic basis for inconsistent soybean yield response to N fertilizer addition commonly reported in the literature (e.g., Wortman et al., 2012; Mourtzinis et al., 2018). Estimation of indigenous soil N supply would also be useful in future research aimed at evaluating the yield response to different combinations of N-fertilizer type, amount, placement depth, seasonal timing, and economic profitability (e.g., Salvagiotti et al., 2009; McCoy et al., 2018). Moreover, opportunities to ameliorate N limitation in soybean by implementing practices that may enhance indigenous soil N supply (e.g., cover crops, use of green or animal manures, etc.) and options to increase N_2 fixation can also be explored.

While the amount of N fertilizer applied in our study was far from being economically feasible or environmentally sound, our findings provide a first step towards better identifying environments in which soybean N limitation is expected to be large (i.e., high-yield environments with relatively low indigenous soil N supply). Because measured maize grain yield in the N-omission plot was a good predictor of indigenous soil N supply, whereas individual soil properties were not, as documented in our study and in prior studies for cereal crops (e.g., Cassman et al., 1996; Wortmann et al., 2011), a soybean producer in the US Corn-Soybean Belt might consider not applying N-fertilizer on a few strips in the maize field (during a 2-y maize-soybean rotation) to potentially estimate the field's indigenous soil N supply. The measured grain yield in those N-omission maize strips could be used as a proxy indication of indigenous soil N supply when coupled with the relationship shown in Fig. 4b. If complemented with estimates of soybean yield potential (derived from crop models, or based on soybean yields achieved in previous years, such an approach would allow producers to identify fields in which there may be a large N limitation and thus make more informed decisions about management practices to reduce the N limitation in high-yield soybean production systems.

3.5. Conclusions

This study investigated the physiological drivers for greater seed yield and protein concentration in the full versus zero N treatments across 13 high-yield soybean production environments in USA and Argentina. Seed yield and protein concentration were higher in the full-N versus zero-N treatment due to greater accumulated N without changes in NUE and NHI, when ADM was expressed as glucose equivalents (ADM_e) to account for differences in seed biomass composition. Indigenous soil N supply modulated the magnitude of the (full-N minus zero-N) seed yield difference, indicating that the N limitation is largest in high-yield environments with relatively small indigenous soil N supply.

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3.7. Supplementary material

Table 3-S1. Description of the 13 high-yield field experiments conducted in Balcarce, Argentina (BA) and Nebraska, USA (NE).

Site	Crop season	Location	Variety (and MG)	Sowing date(s)	Previous crop	Precipitation^{a} (mm)	Irrigation^{a} (mm)	Fertilizer N applied (kg N ha^{-1})^{b}	Indigenous soil N (kg N ha^{-1})^{c}	Tillage method
Balcarce, BA	2014/2015	37.765 S 58.313 W	DM2200 (MG:2.1),	Nov 1, Nov 27	Wheat	367	175	640	133	Disk
			DM3810 (MG:3.8),			350	175	540	156	
			DM4612 (MG:4.6).							
Mead, NE	2016†	41.244 N 96.502 W	AG2723 (MG:2.7)	May 8	Maize	426	51	870	98	Strip-till
	2017	41.248 N 96.486 W	AG2723 (MG:2.7)	May 6	Maize	256	95	870	63	Strip-till
Saronville, NE	2016†	40.601 N 97.966 W	AG2431 (MG:2.4)	April 26	Maize	230	193	870	150	Disk
	2017	40.596 N 97.960 W	P33T72R (MG:3.3)	April 23	Maize	311	112	870	72	Strip-till
Smithfield, NE	2016†	40.538 N 99.683 W	P24T19 (MG:2.4)	May 13	Maize	329	84	870	139	No-till
	2017	40.538 N 99.686 W	P27T59 (MG:2.4)	May 25	Maize	202	86	870	186	No-till
Atkinson, NE	2017	42.658 N 99.029 W	AG2723 (MG:2.7)	April 24	Maize	362	410	870	208	Disk

[a] Total precipitation and irrigation between crop emergence and physiological maturity (R7 stage). [b] Total fertilizer N amount applied to the full-N treatment.

[c] Indigenous soil N supply estimated from N accumulation in aboveground dry matter in a N-omission maize plot adjacent to each soybean experiment.

Table 3-S2. Correlation matrix for soil properties measured at sowing (clay fraction, soil organic carbon [SOC; %], inorganic N-NO_3^- [kg N ha^{-1}], and SOC-to-clay fraction ratio), total irrigation water N (kg N ha^{-1}), and indigenous soil N supply (ISNS; kg N ha^{-1}). ISNS was estimated from accumulated N aboveground dry mater in the N-omission maize plots located contiguous to the soybean experiments. Pearson's correlation (r) and associated p values are shown below and above the diagonal, respectively.

	ISNS	Clay fraction	SOC	Irrigation N	Soil N-NO_3^-	SOC:clay ratio
ISNS	1	0.06	0.86	0.26	0.3	0.55
Clay fraction	-0.68	1	0.32	0.7	0.06	0.11
SOC	0.07	-0.4	1	0.91	<0.01	<0.01
Irrigation N	0.45	0.16	0.05	1	0.72	0.99
Soil N-NO_3^-	0.42	-0.69	0.91	0.15	1	<0.01
SOC:clay ratio	0.25	-0.61	0.97	0.01	0.97	1

CHAPTER 4: DRIVERS OF NITROGEN LIMITATION IN HIGH-YIELD SOYBEAN

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Abstract

The physiological pathways underpinning high-yielding soybean (*Glycine max* L. Merr.) nitrogen (N) limitation for seed yield and protein concentration are unknown. The objective of this study was to identify physiological mechanisms underlying N limitation in soybean during the growing season and their effect upon seed yield and protein concentration. A soybean crop supplied only by indigenous soil N supply and biological N_2 fixation (zero N) was compared to a crop that received ample N supply in the form of N fertilizer (full N). Accumulated N and aboveground dry matter (ADM), crop growth rate, and N accumulation rate, remobilized ADM and N, leaf area index (LAI), leaf photosynthesis, and fraction of absorbed photosynthetically active radiation (fAPAR) were measured in seven high-yield environments (5.3-6.7 Mg ha^{-1}). The full N treatment reached maximum fAPAR four days prior to the zero N treatment owing to greater LAI. Consequently, greater ADM (in full N) between the beginning of pod setting (R3) and full seed (R6) stages generated larger numbers of seed. Up to 62% of the N accumulated prior to the beginning of seed filling (R5 stage) was remobilized to seed in both treatments. However, the full N treatment accumulated an extra 45 kg N ha^{-1} before R5 and, therefore, was able to remobilize more 26 kg N ha^{-1} to the seeds than the zero N treatment; this increased N supported greater seed weights and seed N concentrations. Findings from this study indicate that future efforts to reduce N limitation in soybean while maintaining seed quality should focus on enhance biological N_2 fixation and/or indigenous soil N supply prior to the beginning of seed filling.

Keywords: Soybean, Glycine max (L.) Merr., nitrogen, crop growth rate, nitrogen uptake rate

4.1. Introduction

Soybean [*Glycine max* (L.) Merr.] is the world’s most important legume crop and source of vegetable protein for food and feed (Wilson, 2008). Its nitrogen (N) requirement per unit of photosynthate produced is one of the highest among food crops (Sinclair and de Wit, 1975). On average, soybean requires 80 kg N in aboveground dry matter (ADM) per metric ton of seed produced (Salvagiotti et al., 2008; Tamagno et al., 2017). This value is about 3 times higher than the N requirement per unit of grain in cereal crops such as rice, wheat, or maize (Setiyono et al., 2011; Yin et al., 2019; Barraclough et al., 2010). Unlike cereal crops, fertilizer N is rarely used on soybean, except for an occasional small amount applied as ‘starter’ at sowing. Hence, soybean relies on two major N sources to meet its large N requirement: (i) biological N_2 fixation, and (ii) N from soil organic matter mineralization, atmospheric dry and wet deposition, water table, and irrigation water (hereafter collectively called ‘indigenous soil N supply’). However, unclear is whether the N from fixation and indigenous soil N supply is sufficient to meet soybean N requirement as average yield continues to increase due to improved cultivars and agronomic practices (Specht et al., 2014; Grassini et al., 2014).

Quantifying a N limitation in soybean is challenging for two reasons. First, it is difficult to eliminate every yield-limiting factor besides N supply during the entire growing season so that the N limitation can be assessed in crops grown in near optimal conditions, which in turn, would allow greater expression of the inherent yield potential of modern soybean cultivars. Second, proper synchronization between N supply and crop N demand is challenging in soybean because application of N fertilizer reduces N fixation (Streeter and Wong, 1988). To overcome these challenges, Cafaro La Menza et al. (2017, 2019) developed a protocol for analyzing N limitation in soybean crops grown across a wide range of environments, sowing dates, and cultivar maturity groups (MGs), resulting in a range of seed yields from 2.5 to 6.7 Mg ha^{-1}. In all cases crops were irrigated and optimally managed to avoid nutrient limitations (except for N), and to avoid incidence of weeds, pathogens, and insect pests. The protocol consisted of a side-by-side comparison between a ‘control’ treatment (hereafter called ‘zero-N’) that forces the crop to rely on biological N_2 fixation and site-specific indigenous soil N supply, and a ‘full-N’ treatment designed to provide the crop with sufficient fertilizer N supply to optimally match seasonal crop N demand. With timing and amount of N fertilizer applications adjusted to in-season crop N requirements, the full-N treatment was designed to eliminate N limitation at any time of the growing season and help to overcome the typical trade-off between biological N_2 fixation and indigenous soil N supply.

The set of studies by Cafaro La Menza et al. (2017, 2019) confirmed that N supply is limiting in high-yield soybean production environments. Furthermore, the magnitude of this limitation decreases with increases in the amount of indigenous soil N supply. They showed that higher seed yields in the full versus zero N treatment were associated with greater N accumulation in ADM, seed number, seed weight, and seed N concentration. In contrast, there were no detectable differences in harvest index, N-use efficiency, N-harvest index, or seed oil concentration between N treatments. However, these studies did not provide insight about the dynamic changes in physiological mechanisms over time that eventually lead to end-of-season R7-based differences in seed yield and protein concentration. For example, it would be of scientific interes to determine the underlying mechanisms explaining the observed differences in aboveground biomass, seed number, and seed weight as a result of contrasting N supplies at various time points in crop development. Such an assessment would be useful for a more mechanistic understanding of the N limitation in soybean, and to further inform management practices that aim to overcome this limitation and sustain future yield gains while maintaining seed protein concentration.

Considering the need to increase current soybean yield on existing cropland area to meet future demand while avoiding further conversion of natural ecosystems into cropland, it is important to better understand the in season physiological drivers that underlie the N limitation in soybean. The objective of this study was to elucidate the underlying mechanisms (e.g. leaf area index, leaf and canopy photosynthesis, remobilized N to the seed) that explain differences in seed yield and protein concentration between contrasting N supply levels. To do this, we used a set of high-yield irrigated soybean experiments conducted during 2017 and 2018 in farmer fields in Nebraska. These experiments included detailed weekly measurements of accumulated dry matter and N and other physiological processes during the entire crop season, allowing a comprehensive assessment of the N limitation in soybean.

4.2. Materials and methods

4.2.1. Experimental sites and design

This paper presents new data from the set of experiments conducted by Cafaro La Menza et al. (2017;2019) in Nebraska (NE), USA (Table 4-1). These studies addressed the questions of: is soybean yield limited by N supply? and are the differences in seed yield and protein concentration due to ample N supply explained by changes in N uptake, N-use efficiency, and/or N harvest index? Nebraska is the fifth largest US soybean producing state, including the largest area with irrigated soybean in the world (ca. 2.6 million ha) where producers achieve average irrigated yields of 4.4 Mg ha^{-1} (USDA-NASS, 2014-2018; https://www.nass.usda.gov/). This yield level is much higher than the average yields in other major soybean producing areas (ca. 3 Mg ha^{-1}) that include the US

Corn Belt, the Brazilian Cerrados, and Argentinean Pampas, where most production is rainfed. Hence, the NE irrigated production environment provides a suitable background to evaluate the N limitation in high-yield soybean. Our experiments were conducted in pivot-irrigated producer fields located at four sites across NE (Mead, Saronville, Smithfield, and Atkinson) during two crop seasons (2016 and 2017). All fields in these sites have consistently produced high soybean yields in previous years (>5 Mg ha^{-1}). This combination of site-years well represents the range of weather, soils, and management practices (e.g., tillage methods, cultivar maturity groups [MGs]) that occur across the state of NE (Table 4-1; Figure 4-1). We excluded the experiments in Atkinson in 2016 due to a severe powdery mildew (*Microsphaera diffusa*) infestation. A portable weather station was erected in each experiment site both years (2016 and 2017) to monitor hourly soil and air temperature, precipitation, relative humidity, and wind speed. A well-validated soybean crop model (SoySim; Setiyono et al., 2010) was used to simulate the yield potential for each site-year using local weather and producer-reported sowing date and cultivar MG at each site-year (Table 4-1). In all cases, simulated yields exceeded the yield threshold of 4.5 Mg ha^{-1} proposed in the literature to define high-yield production environments where N limitation in soybean might occur (Salvagiotti et al., 2008).

Each site-year combination (total of seven) is referred hereafter to as an ‘environment’ that included two N treatments (full-N and zero-N) in a complete randomized design with four replicates per treatment (size: 176 m^2 each). Experimental plots were purposely placed in areas within each field where the greatest (maize and/or soybean) yields had been achieved in previous years. These soybean crops were always grown in a 2-y rotation with maize, which is the dominant practice in the US Corn belt (Grassini et al., 2014). Soils were deep, without physical or chemical constraints to root growth. Soil series at the NE field sites were O’Neil sandy loam (Atkinson), Yutan silty clay loam (Mead), Hastings silt loam (Saronville), and Holdrege silt loam (Smithfield). Crops were grown with the explicit goal of removing any yield-limiting factor (except for N in the zero-N treatment). In all cases, seeds were treated with fungicide and insecticide, but received no inoculant that is a common management practice for soybean-maize rotations in the US Corn Belt, and the commonly detected lack of yield response to inoculation in high-yield environments occurs (de Bruin et al., 2010; Leggett et al., 2017 and references cited therein). Row spacing was 0.76 m in all environments, with seeding rates well above the recommended plant density to maximize soybean yields (De Bruin and Pederson, 2009). Soil water content in the upper meter was monitored using Watermark® sensors and maintained above 50% of available water throughout the entire growing season, except for a short period in Atkinson (Figure 4-1). Several prophylactic foliar applications of herbicide, fungicide, and insecticide, and pre-sowing nutrient applications (based on soil test results) kept the crops free from biotic and nutrient stresses (besides N in the zero N treatment). Inorganic N at sowing in the upper 60 cm ranged from 25 to 58 kg N ha^{-1}.

Table 4-1. Description of the seven high-yield field experiments conducted in Nebraska, USA (NE). K:1M NH4-acetate extractable potassium (K). P: Bray-1 phosphorous; MG: maturity group; SOC: soil organic carbon; S: sulfur; Ca: calcium; Mg: magnesium; Zn: zinc.

Site	Crop season	Cultivar (and MG)	Sowing date	Yield potential (Mg ha^{-1})	Tillage method	pH	SOC (g kg^{-1})	Soil P (ppm)	Soil K (ppm)	Seeding rate (m^{-2})	Applied fertilizer (kg nutrient ha^{-1}) ‡
Mead (41.244 N; 96.502 W)	2016	AG2723 (MG 2.7)	May 8	6.4	Strip till	6.3	22	12	335	25	N (20); P (21); S (17); Zn (2)
	2017	AG2723 (MG 2.7)	May 6	6.4	Strip till	6.7	24	25	268	35	N (12); P(25)
Saronville (40.601 N; 97.966 W)	2016	AG2431 (MG 2.4)	April 26	5.5	Disk	6.2	21	17	395	25	N (17); P (35); S (10); Zn (1)
	2017	P33T72R (MG 3.3)	April 23	5.6	Strip till	7.1	20	33	438	35	N(6); P(20)
Smithfield (40.538 N; 99.683 W)	2016	P24T19 (MG 2.4)	May 13	5.7	No till	7.5	16	12	487	32	P (38); S (1)
	2017	P27T59 (MG 2.7)	May 25	4.8	No till	7.2	18	25	373	44	N (24); P (48); S (27)
Atkinson (42.658 N; 99.029 W)	2017	AG2723 (MG 2.7)	April 24	6.3	Disk	7.0	14	7	184	40	N (20); P (22); K (50); Ca (62); Mg (8); S (33); Zn(2)

K: 1M NH4-acetate extractable potassium (K). P: Bray-1 phosphorous; MG: maturity group; SOC: soil organic carbon; S: sulfur; Ca: calcium; Mg: magnesium; Zn: zinc.

Measurements depth of SOC, pH, P, and K was 60 cm.

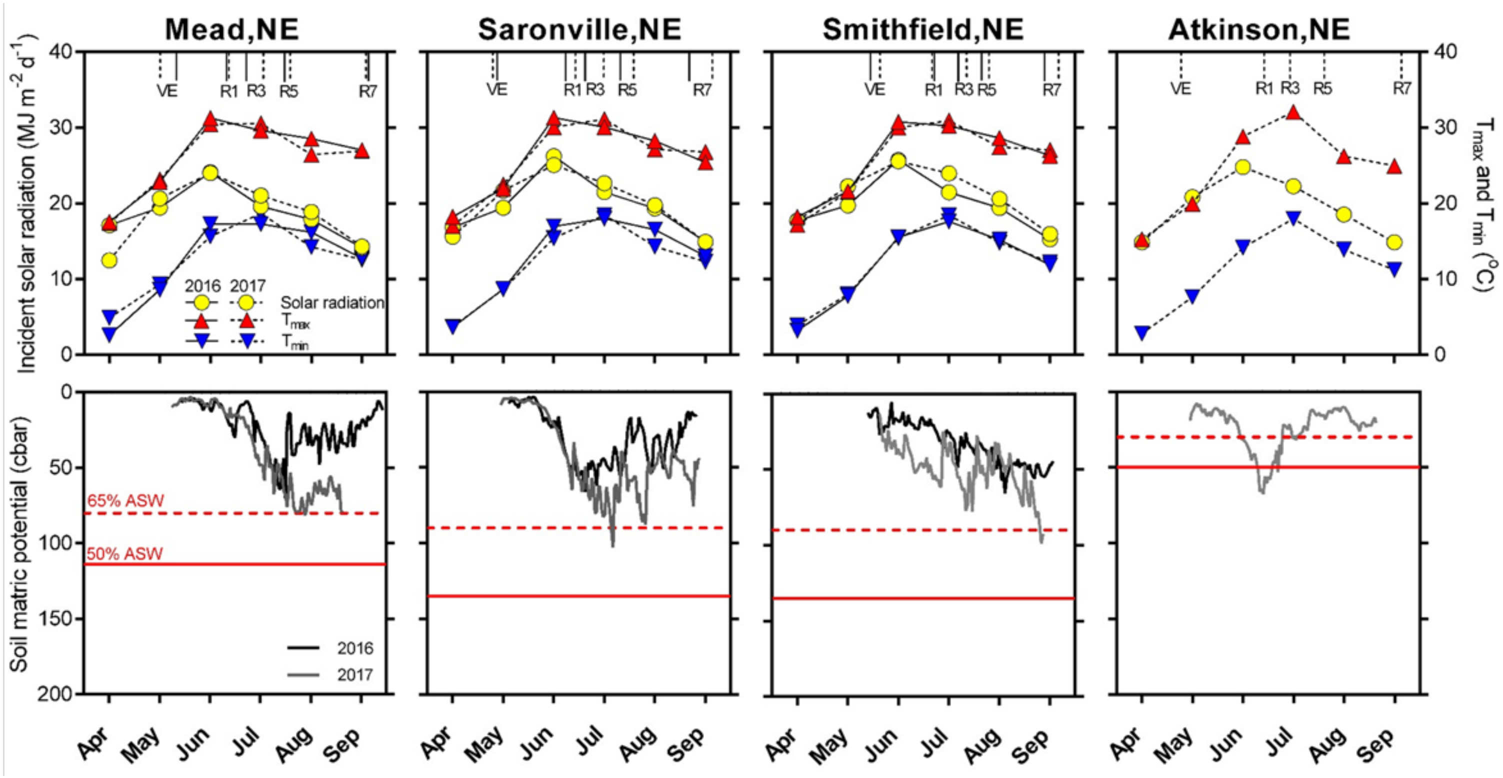

Figure 4-1. Monthly average incident solar radiation, maximum (Tmax) and minimum temperature (Tmin) (upper panels) and soil matric potential in the upper meter (bottom panels) during the growing season in the seven experiments conducted in 2016 (solid line) and 2017 (dashed line). Arrows in top x-axis indicate average dates of emergence (VE), beginning of flowering (R1), beginning of pod setting (R3), beginning of pod filling (R5), and physiological maturity (R7). Ticks in the x-axis represent the mid-point of each month. To provide a reference, the soil matric potential that corresponds to 65% and 50% of plant available soil water (ASW) is indicated with dashed and solid horizontal lines, respectively (Irmak et al., 2014). Note that values were higher in Atkinson as a result of coarser soil texture.

4.2.2. Nitrogen treatments

Two N treatments were compared in each environment: (i) a ‘zero N’ treatment in which the crop relied on indigenous soil N supply and biological N_2 fixation and (ii) a ‘full N’ treatment designed to provide the crop with sufficient fertilizer N to optimally match seasonal crop N demand. The N fertilizer was applied as urea and broadcast between plant rows in the full-N treatment. A total seasonal amount of 870 kg N ha^{-1} was applied at all sites based on (i) site-specific yield potential simulated using the SoySim model (Setiyono et al., 2010), (ii) N uptake requirement of 80 kg N per Mg^{-1} seed yield (Salvagiotti et al., 2008; Tamagno et al., 2017), and (iii) fertilizer recovery efficiency of 70% to account for unavoidable N losses. Total N fertilizer amount was split into five crop stage dependent applications to optimally match the expected increase in crop N requirement during the crop season (Thies et al., 1995; Bender et al., 2015). We applied 10%, 10%, 20%, 30%, and 30% of the total N fertilizer at the V2, V4, R1, R3, and R5, stages of soybean development respectively. The splitting of total of the total 870 kg N ha^{-1} into five smaller applications was a choice based on the assumption that this protocol might neutralize any trade-off involving a depression in crop N uptake from N_2 fixation as a result of N fertilizer application by providing enough N to meet crop N requirements irrespective of the contribution from N fixation.

4.2.3. Field measurements

The seven experiments included detailed measurements of phenology, leaf area, photosynthesis, and accumulated ADM and N during the entire crop season. We used the phenological stages defined by Fehr and Caviness (1977) to track crop phenology. According to this scheme, VE corresponds to emergence and each Vn thereafter to the nth node on the main stem with fully developed leaves, beginning with the unifoliolate node (V1). In the case of reproductive stages (Rn), the R1 stage corresponds to the beginning of blooming and is visually detected when there is one open flower at any node on the main stem, the R3 stage corresponds to the beginning of pod setting and is visually detected when there is a pod of 5-mm length at one of the four uppermost nodes of the main stem with a fully developed leaf, and the R5 stage corresponds to the beginning of seed filling and it is visually detected when there is a seed of 3-mm length in a pod at one of the four uppermost nodes on the main stem with a fully developed leaf. Finally, the R7 stage corresponds to physiological maturity and is visually detected when at least one of the pods on a plant has reached maturity color. Crop development stages were recorded from ten consecutive plants within one row in each replicate for all treatments and environments every seven days, starting at VE and concluding at R7. Following other studies (e.g., Lindquist et al., 2004), crop phenological development was made comparable across experiments (with different temperature regimes, sowing date, and cultivar MG) by defining developmental stage (DVS) using a dimensionless scale where 0, 1, and 2 correspond to the VE, R3, and R7 stages, respectively. In our case, development rate was calculated based on daily mean air temperature using a beta function as defined by Wang and Engel (1998), with appropriate cardinal temperatures for each phase as reported by Setiyono et al. (2007). In our scale, DVS values of 0.5 and 1.5 would mean that half of the number of thermal units between VE-R3 and between R3-R7, respectively, have been accumulated. We did not account for photoperiod on our DVS calculation as the four locations were located within a narrow latitudinal band (from 40.5º to 42.6º).

Seasonal dynamics of ADM and accumulated N were assessed in each environment and treatment by collecting a 1-m row (0.76 m spacing between adjacent rows) of consecutive plants (same sampling spot of phenology assessments), surrounded by two rows receiving the same N treatment, in each replicate. We did not attempt to collect root biomass. Samples were collected weekly from VE until R7 and separated into different plant organs (green leaves, stems, seed, pod walls, and senesced leaves). Green leaves (defined as any leaf with >50% green area) were scanned to determine the leaf area index (LAI) in each replicate and environment (LAI-3100 area meter LI-COR, Lincoln, NE). Abscised leaves were also collected every week from a 1-m row net placed in-between rows in each replicate. Plant tissue samples were oven-dried at 70 °C until reaching constant weight. Total ADM was calculated as the dry matter sum of all plant tissue components and included collected abscised leaves. Each plant tissue sample was separately ground in a Wiley mill (1-mm screen mesh), and N concentration was determined with a dry combustion-based analyzer (LECO Corporation, St Joseph, MI). Accumulated N in ADM was calculated based on the ADM and N concentration of each plant tissue and summation of all plant components. Average coefficient of variation (CV) among replicates was ca. 10% for both ADM and accumulated N, indicating that number and size of replicates were appropriate for a robust estimation of these parameters in the two N treatments. Larger plant samples (4.6 m^2) were collected shortly after R7 from the two central rows in each plot, surrounded by two rows receiving the same N treatment, to obtain an end-of-season post-R7 estimate of seed yield. Two sub-samples of 200 seeds from the threshed seed of each plot were weighed to estimate the mean individual seed dry weight and to derive the number of seed per harvested area. Seed yield and seed weight were adjusted to 0.130 kg H_2O kg^{-1} seed, which is the standard moisture content for commercialization in soybean.

Incident and absorbed photosynthetically active radiation (IPAR and APAR, respectively) were measured by installing light sensors in one or two plots in each treatment in each environment. IPAR was measured above the canopy using a point quantum sensor facing up (LI-190SA, LI-COR, Lincoln, NE). Transmitted PAR was measured by the placement of a single-line quantum sensor (LI-191SA, LI-COR, Lincoln, NE) at the soil surface diagonally across rows. Total (canopy plus soil) reflected PAR was measured using an inverted point quantum sensor (identical to the one used for IPAR measurement), facing down, placed 2 m above crop canopy. Reflected PAR from soil was measured using an inverted line quantum sensor (identical to the one used for transmitted PAR measurement), facing down, placed 5 cm above soil surface and diagonally across rows. Measurements were taken every second and recorded as a 30-minute average, starting soon after VE and ending at R7. All sensors were calibrated by the manufacturer and a cross-calibration of all sensors was performed every year before placing sensors in the field and after harvest. Sensors were leveled (if needed) and cleaned every 3 to 5 d. Reflected PAR by crop canopy was calculated as total reflected PAR minus reflected PAR from soil. Daily APAR was calculated as IPAR minus transmitted PAR and reflected PAR from crop canopy, and expressed as fraction of daily IPAR (fAPAR). Finally, radiation-use efficiency (RUE) was estimated as the slope of the relationship between all the accumulated ADM sampling points and their corresponding accumulated APAR from VE to R7.

Leaf photosynthesis was measured at four stages during the growing season (R1, R3, R5, and at a mid-point during seed filling) in all treatments and environment in 2017. Photosynthesis was measured on the central leaflet of the third most recently developed leaf of one plant in each replicate. To ensure representativeness, plants were selected to coincide with the average crop stage measured on the day of the measurement. Light response curves were built by varying the photosynthetic photon flux density (PPFD) levels, from 1800 to 0 µmol photons m^{-2} s^{-1} of a red/blue LED light source of the open-flow gas exchange system (LI-6400, Lincoln, NE). Carbon dioxide (CO_2) level inside the chamber of the open-flow gas exchange system was kept at 400 ppm, leaf temperature was set at 25 ºC, and the minimum measurement waiting time was 60 seconds or until reaching a CV ≤ 3 % of CO_2 assimilation rate.

4.2.4. Data analysis

A logistic model (France and Thornley, 1984) was fitted to portray dynamics of ADM and accumulated N during the crop growing season:

$$\text{ADM or N accumulated} = \frac{W0 * Wf}{W0 + (Wf - W0) * e^{-u*t}} \qquad \text{Eq. (1)}$$

where W0 is the initial ADM (g m^{-2}) or N accumulated (kg ha^{-1}) at emergence, Wf is the maximum ADM or accumulated N during the growing season, t is time in days (d), and u is a constant of proportionality of plant growth and its decaying with time. Analysis of residuals from the fitted model did not reveal any bias during the growing season (r^2 from 0.96 to 0.99). The derivative of Eq. (1) represents the crop growth rate (CGR; g m^{-2} d^{-1}) or the N accumulation rate (NAR; kg N ha^{-1} d^{-1}). Estimating CGR and NAR using this approach helps remove the measurement error associated with specific sampling times and has been used extensively in field research (e.g., Hall et al., 1995; Lindquist et al., 2005; Bange et al., 1997). Similarly, the seasonal dynamics of LAI were modelled using a combination of a sigmoidal equation before peak of LAI and a quadratic model after the maximum LAI was reached as follows:

$$\text{LAIi} = \text{LAIve} + \frac{(\text{LAImax} - \text{LAIve})}{\left(1 + 10^{((c - t) * d)}\right)}$$

$$\text{LAIt0} = \text{LAIve} + \frac{(\text{LAImax} - \text{LAIve})}{\left(1 + 10^{((c - t0) * d)}\right)}$$

$$\text{LAIii} = \text{LAIt0} + a * (t - t0) + b * (t - t0)^2 \qquad \text{LAI} = \text{IF}(t < t0, \text{LAI1}, \text{LAI2})$$

Eq. (2)

where LAIi is the LAI before the maximum LAI (LAImax), LAIve is the LAI at emergence, t is time expressed as days after sowing, c and d are curve-shape parameters, LAIt0 is the LAI at the peak of LAI (t0), LAIii is the LAI after the peak, and a and b are curve-shape parameters.

Seasonal dynamics in fAPAR were compared between N treatments to assess differences in IPAR capture. Because we did not have light sensors in each plot, we used the following approach to estimate daily fAPAR for each experimental unit in each environment. First, we generated a relationship between fAPAR and LAI using all available dates of LAI sampling (Supplementary Figure 4-S1). The extinction coefficient (k) was estimated to be 0.54 across all environments and treatments. Second, we estimated daily fAPAR for each experimental unit based on daily LAI obtained from the fitted models (Eq.2).

Sources of carbon for seed dry matter accumulation during the reproductive phase include new photoassimilates and dry matter remobilization from vegetative biomass (Stephenson and Wilson, 1977; Egli et al., 1985). Apparent dry matter remobilization from non-seed ADM was estimated as the difference in non-seed ADM (including stems, leaves, and pod walls) between R5 and R7. We used the term ‘apparent’ because it should be taken as a semi-quantitative (rather than direct) measure of the amount of non-seed ADM remobilized for seed filling. In the case of seed N accumulation, seeds rely on

N uptake from soil and N fixation during seed filling as well as remobilization from non-seed ADM. Remobilized N was estimated following the same approach as for dry matter remobilization. We did not attempt to account for C and N remobilized from belowground biomass.

The following function was used to quantify the response of net photosynthesis to PPFD:

$$A = \frac{\mathrm{Amax} * (\mathrm{PPFD} - \mathrm{Ic}) * \alpha}{\mathrm{Amax} + (\mathrm{PPFD} - \mathrm{Ic}) * \alpha} \qquad \text{Eq. (2)}$$

where A is the photosynthetic rate in μmol CO_2 m^{-2} s^{-1}, Amax is the maximum photosynthetic rate at light saturation in μmol CO_2 m^{-2} s^{-1}, PPFD is photosynthetic photon flux density in μmol photons m^{-2} s^{-1}, Ic is the light compensation point (μmol photons m^{-2} s^{-1}), and α is the initial slope of the response curve. Dark respiration (Rd) can be estimated from the fitted parameter values as –α*Ic.

We used a combined analysis of variance (ANOVA) to analyze the effect of N treatments across environments (SAS® PROC MIXED v.9.3; Moore and Dixon, 2015). The following parameters were evaluated: seed yield, seed number, seed weight, ADM at R7, accumulated N at R7, seed N concentration, remobilized ADM, and remobilized N. A similar analysis was performed to identify the crop stage(s) with largest difference between treatments on CGR and NAR. To do this, CGRs and NARs obtained per experimental unit were averaged by treatment over four crop phases: VE-R1; R1-R3; R3-R5; R5-R7. The combined ANOVA assumes homogeneity within and between environment's variances. Indeed, variances were not too different (Fmax < 6); therefore, our analysis can be considered robust (Milliken and Johnson, 2009). In this analysis, each site-year combination was referred to as an environment. Both environments and N treatments were treated as fixed effects. In this analysis, environments cannot be directly compared, but the analysis can provide an estimate of the overall significance and consistency of the differences between N treatments across environments. Treatment differences were evaluated using contrasts.

Differences in the parameters of the light response curve (Amax, Ic, α, Rd) between N treatments were performed using mixed models (InfoStat; Di Rienzo et al., 2011). Linear regression analysis was used to investigate relationships and trade-offs between the variables measured. Slope, intercept, and coefficient of determination (r^2) of linear regressions were calculated and differences on the measured variable between treatments were tested with F tests. To remove the confounding effect of differences in phenology across environments, seasonal patterns in ADM, accumulated N, CGR, NAR, and LAI are shown as a function of the DVS calculated for each environment and data are pooled for each N treatment to facilitate the comparison.

4.3. Results

4.3.1. Impact of soybean nitrogen limitation on seed yield components

Soybean seed yield ranged from 5.3 to 5.8 Mg ha^{-1} (zero N) and from 5.5 to 6.7 Mg ha^{-1} (full N) across experiments (Table 4-2). Average seed yield was 0.6 Mg ha^{-1} greater in the full than in zero N treatment (Table 4-2 and 3). In all cases, measured yields in the N full treatment were within ±15% of simulated yield potential. On average, accumulated N was 51 kg N ha^{-1} higher in the full versus zero N treatments (446 versus 395 kg N ha^{-1}, respectively), which translated to 10% greater ADM at R7 in the full versus zero N treatments (13.2 versus 12.0 Mg ha^{-1}, respectively). The full N treatment exhibited higher seed number (7%; 3301 versus 3096 seeds m^{-2}) and seed mass (4%; 185 versus 179 mg) compared with the zero N treatment. As expected, there was a trade-off between seed weight and seed number (Figure 4-2, Table 4-2). The slope of the linear regression between seed weight and seed number was different ($p=0.005$) between the zero and full N treatment (-0.02 versus -0.05 mg per additional seed, respectively), suggesting that the trade-off was alleviated in the case of the full N treatment. Interestingly, the zero N treatment attained seed yields ca. 5.5 Mg ha^{-1} through different combinations of seed number and weight, suggesting that irrigated soybean in NE relying exclusively on N supply from soil and N fixation has an upper yield limit around this value. In contrast, in four of the seven environments, seed yield in the full N treatment reached or exceeded 6 Mg ha^{-1} due to a combination of higher seed number and weight. In the other three cases, the yield differential between the full and zero N was smaller (<0.5 Mg ha^{-1}), which was attributable to the high indigenous soil N supply at these three site-years as documented in a previous study (Cafaro Le Menza et al., 2019).

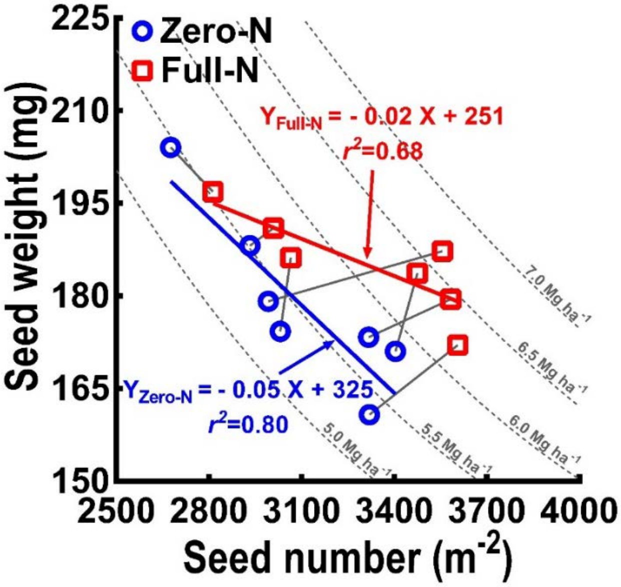

Figure 4-2. Observed coordinates for soybean seed number and seed weight at physiological maturity in the full (red squares) and zero-N (blue circles) treatments in the seven environments. Connecting grey lines relate paired treatments from the same environment. Dotted lines indicate 0.5 Mg ha^{-1} in seed yield that reflect correspondence of a given yield with the seed number and weight coordinates. Parameters of the fitted linear regressions (solid blue and red lines for zero and full-N treatment respectively) and coefficient of determination (r^2) are also shown. Note that regression lines do not imply causality (with respect one trait versus the other); instead, they are shown to illustrate that the trade-off between seed weight and seed number varies between N treatments in terms of where the paired coordinates are positioned in the graph.

Table 4-2. Mean soybean seed yield and yield components (seed number and weight), aboveground dry matter (ADM), accumulated nitrogen (N) in ADM, and seed N concentration at physiological maturity (R7 stage), and remobilized ADM and N from non-seed ADM in the two N treatments across environments.

Location	Year	Treatment	Seed yield ($Mg\ ha^{-1}$)	Seed number (m^{-2})	Seed weight (mg)	ADM at R7 ($Mg\ ha^{-1}$)	Accumulated N at R7 ($kg\ N\ ha^{-1}$)	Seed N concentration ($g\ N\ kg^{-1}$)	Remobilized ADM ($Mg\ ha^{-1}$)	Remobilized N ($kg\ N\ ha^{-1}$)
Mead	2016	zero	5.3	3320	161	12.8	419	56.8	1.0	154
		full	6.2	3606	172	13.7	460	57.1	1.5	174
	2017	zero	5.4	2992	179	11.3	370	56.7	1.9	147
		full	6.7	3557	187	13.1	459	60.0	2.5	179
Saronville	2016	zero	5.8	3405	171	12.1	408	57.7	0.9	134
		full	6.4	3476	184	13.4	436	58.0	1.0	140
	2017	zero	5.7	3317	173	13.1	401	58.0	2.1	167
		full	6.4	3584	179	14.7	485	62.7	1.6	196
Smithfield	2016	zero	5.3	3031	174	10.4	364	54.7	1.7	130
		full	5.7	3065	186	11.5	384	57.5	1.1	157
	2017	zero	5.5	2676	204	11.2	367	62.3	1.5	151
		full	5.5	2813	197	12.4	432	64.5	1.0	172
Atkinson	2017	zero	5.5	2933	188	13.1	436	61.2	1.5	165
		full	5.7	3009	191	13.3	466	61.6	1.8	214

Table 4-3. Analysis of variance for effect of nitrogen (N) treatment on soybean seed yield and its components (seed number and weight), aboveground dry matter (ADM), accumulated nitrogen (N) in ADM, and seed N concentration at physiological maturity (R7 stage), and remobilized ADM and N from non-seed ADM. Each of the 7 location × year combinations was considered to be a separate environment (E) for the N × E interaction in this analysis. Also shown are the F-test values and probabilities for the fixed effects, mean squares (MS) for the random effects, estimated means for each treatment, and a test of whether the treatment mean difference was significantly different from zero.

		Seed yield	Seed number	Seed Weight	ADM at R7	Accumulated N at R7	Seed N concentration	Remobilized ADM	Remobilized N
Fixed effects	d.f.	F	F	F	F	F	F	F	F
E	6	7.3***	20.4***	59.2***	16.1***	5.9**	4.6**	5.3**	5.0**
N	1	42.6***	18.7***	24.8***	16.8***	27.7***	5.6*	0.2	16.8***
N x E	6	3.0*	2.2	4.1**	0.5*	1.2	0.3	2.9*	0.6
Random effects		MS	MS	MS	MS	MS	MS	MS	MS
Rep (E)	21	0.08	31207	15.7	0.5	1011	11	0.25	566
N x Rep (E)	21	0.12	31437	24.1	1.3	1326	10	0.17	578
Estimated means		(Mg ha^{-1})	(m^{-2})	(mg)	(Mg ha^{-1})	(kg N ha^{-1})	(g N kg ADM^{-1})	(Mg ha^{-1})	(kg N ha^{-1})
Full-N		6.1	3301	185	13.2	446	60	1.5	176
Zero-N		5.5	3096	179	12.0	395	58	1.5	150
Difference (full-N -zero-N)		0.6***	205***	7***	1.2***	51***	2*	nil	26***

Asterisks indicate statistical significance at *p<0.05, **p<0.01, and ***p<0.001.

4.3.2. Differences in seasonal accumulated ADM and N between treatments

Accumulated ADM and N followed sigmoidal patterns, with inflection points (peak of CGR and NAR) occurring around DVS = 1.25, which roughly corresponded to the R5 stage. Greater ADM and accumulated N at R7 in the full versus zero N treatments were associated with higher CGR and NAR in the full N treatment from emergence until (but not after) the R5 (Figure 4-3; Table 4-3 and 4). Maximum CGR was slightly higher in the full versus zero N treatment (23.9 versus 22.8 g m^{-2} d^{-1}; p-value = 0.046), but the growth stage at which the peak CGR occurred did not differ among treatments (p-value = 0.212). In contrast, maximum NAR was similar between N treatments (8.2 versus 7.8 kg N ha^{-1} d^{-1} in the full and zero N, respectively; p-value = 0.180), and peak NAR occurred earlier (4 d) in the full compared with the zero N treatment (p-value = 0.001). The CGR and NAR between VE-R5 were 11% and 22% higher in the full versus zero N treatment, respectively. Overall, these differences represented an additional 676 kg ADM ha^{-1} and 45 kg N ha^{-1} accumulated before R5 in the full N treatment. In contrast, differences in CGR and NAR between N treatments were not observable after R5 (p = 0.772 and p = 0.354, respectively; Table 5).

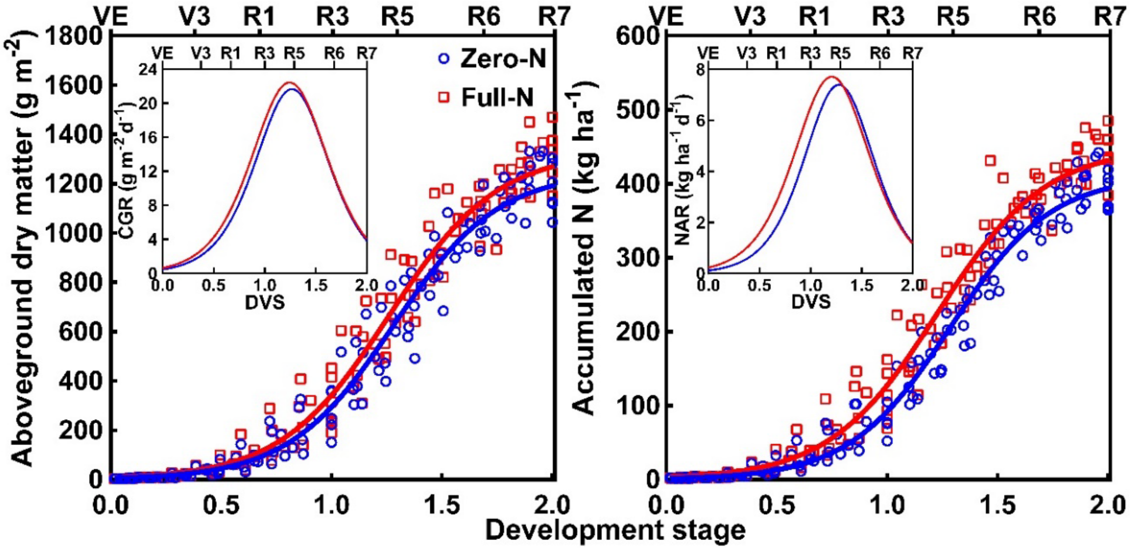

Figure 4-3. Soybean aboveground dry matter and nitrogen (N) accumulation in the full (red squares) and zero-N (blue circles) treatments as a function of development stage (DVS). Stages based on Fehr and Caviness (1977) are shown in the top x-axis. Solid lines represent the fitted Eq. 1 for the full (red) and zero N treatment (blue) based on the pooled data across experiments. Insets show daily crop growth rates (CGR) and N accumulation rates (NAR) for each N treatment. Coefficient of determination (r^2) of fitted models was >0.90 in all cases.

Table 4-4. Mean crop growth rate (CGR) and nitrogen accumulation rate (NAR) for four crop phases: emergence to flowering (VE-R1), flowering to beginning of pod setting (R1-R3), beginning of pod setting to beginning of pod filling (R3-R5), and beginning of seed filling to physiological maturity (R5-R7) in the two N treatments.

Location	Year	Treatment	CGR ($g m^2 d^{-1}$)				NAR ($kg ha^{-1} d^{-1}$)			
			VE-R1	R1-R3	R3-R5	R5-R7	VE-R1	R1-R3	R3-R5	R5-R7
Mead	2016	zero	3.0	8.9	17.1	12.6	1.0	3.1	6.2	4.0
		full	3.3	9.6	18.5	13.0	1.3	3.7	6.5	3.9
	2017	zero	1.3	8.3	19.0	12.5	0.4	2.5	5.8	4.3
		full	1.7	9.9	20.6	13.6	0.7	3.5	6.9	4.6
Saronville	2016	zero	1.9	9.2	19.5	14.1	0.5	3.0	8.0	4.4
		full	2.2	10.3	21.7	15.0	0.9	3.9	7.5	4.5
	2017	zero	2.1	12.1	21.4	12.6	0.6	3.5	6.2	4.2
		full	2.6	13.3	22.1	13.9	0.9	4.7	7.6	4.3
Smithfield	2016	zero	1.5	7.9	17.9	14.8	0.5	3.0	6.7	5.0
		full	2.2	10.0	19.7	14.9	0.7	3.9	8.0	4.5
	2017	zero	2.8	12.4	22.0	12.5	0.9	3.9	7.0	4.1
		full	3.2	13.6	22.1	11.5	1.2	5.1	7.7	3.7
Atkinson	2016	zero	3.6	14.3	20.0	8.9	1.1	4.1	6.0	3.4
		full	4.2	17.3	21.0	7.3	1.6	6.2	7.3	2.5

Table 4-5. Analysis of variance for effect of nitrogen (N) treatment on soybean crop growth rate (CGR) and N accumulation rate (NAR) for the development periods between emergence (VE), flowering (R1), beginning of pod setting (R3), beginning of pod filling (R5), and physiological maturity (R7) in the two N treatments across environments. Each of the 7-location × year x sowing date x variety maturity group combinations was considered a separate environment (E) for the N × E interaction in this analysis. Also shown are the F-test values and probabilities for the fixed effects, mean squares (MS) for the random effects, estimated means for each treatment, and a test of whether the treatment mean difference was significantly different from zero.

		CGR				NAR			
Crop phase		VE-R1	R1-R3	R3-R5	R5-R7	VE-R1	R1-R3	R3-R5	R5-R7
Fixed effects	*d.f.*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
E	6	91.7***	49.7***	9.2***	15.9***	55.6***	20.5***	2.5	7.5***
N	1	38.8***	24.0***	6.8*	0.1	81.7***	105.4***	9.9***	0.9
N x E	6	0.8	0.9	0.3	0.5	0.6	2.8*	1.0	0.7
Random effects		MS	MS	MS	MS	MS	MS	MS	MS
Rep (E)	21	0.06	1.1	2.0	2.5	0.01	0.23	0.92	0.38
N x Rep (E)	21	0.08	1.4	3.3	5.5	0.02	0.17	0.91	0.55
Estimated means		(kg m^2 d^{-1})				(kg N ha^{-1} d^{-1})			
Full-N		2.8	12	20.8	12.7	1.0	4.4	7.4	4.0
Zero-N		2.3	10.5	19.6	12.5	0.7	3.3	6.6	4.2
Difference (full-N -zero-N)		0.5***	1.5***	1.3*	0.2	0.3***	1.1***	0.8***	-0.2

Asterisks indicate statistical significance at *$p<0.05$, **$p<0.01$, and ***$p<0.001$

4.3.3. Drivers for differences in seed number between N treatments

Seed number was associated with the accumulated ADM between R3 and R6 stages (Figure 4-4a). Accumulated ADM during this crop phase had better explanatory power ($r^2 = 0.73$) compared with other crop phases such as R3-R5 or R5-R7 ($r^2 \leq 0.62$). No differences in slope or intercept of this relationship were detected among N treatments ($p = 0.682$ and $p = 0.577$, respectively), indicating that the number of seeds set per unit of accumulated ADM between R3 and R6 was equivalent among N treatments. In contrast, accumulated ADM between R3 and R6 was greater in full compared to the zero N treatment (816 versus 785 g m^{-2}, respectively; paired T-test, $p = 0.045$) (Figure 4-4b). In other words, greater seed number in the full versus zero N treatment was due to greater accumulated ADM during the critical period for seed number determination. Since the duration of the R3-R6 phase was almost identical between N treatments in a given environment ($p = 0.356$), differences in accumulated ADM between N treatments were associated with higher CGR during this crop phase ($p = 0.086$).

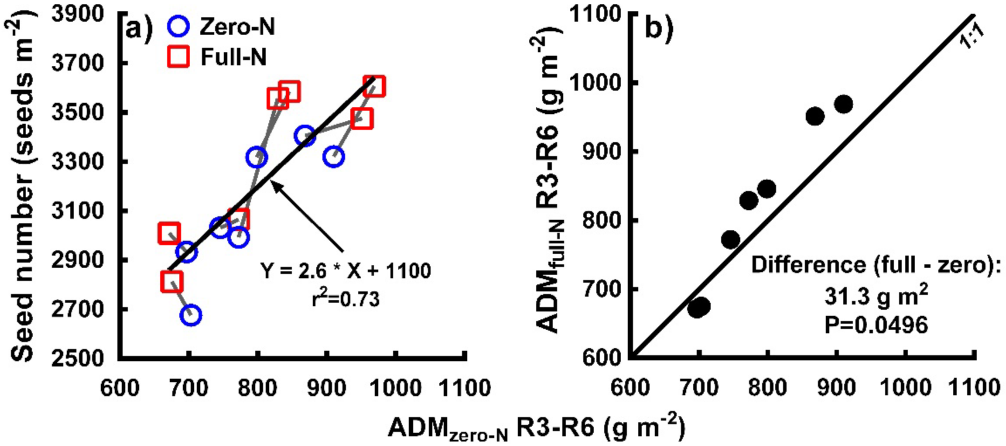

Figure 4-4. (a) Relationship between seed number and accumulated aboveground dry matter (ADM) between the beginning of pod setting (R3) to full seed stage (R6) in the full-N (red squares) and zero-N (blue circles) treatments. Connecting lines relate paired treatments from the same environment. Parameters of the fitted linear regression (solid black line) and coefficient of determination (r^2) are shown. **(b)** Comparison of accumulated ADM between the R3–R6 phase between the full and zero-N treatments.

4.3.4. Mechanisms explaining differences in accumulated ADM between N treatments

There were three key differences in the pattern seasonal LAI between the two N treatments (Figure 4-5a). First, leaf area development was faster early in the season in the full versus zero N treatments, which lead to a consistently greater LAI in the former treatment until the R5 stage (area under the LAI curve up to R5 of 2.76 versus 2.50 in the full and zero N treatment, respectively; p=0.010). Second, while maximum LAI was almost identical between N treatments (5.8 and 5.7 in the full and zero N treatments, respectively; p=0.804), it was reached earlier in the full versus zero N treatment (DVS 1.26 versus 1.34, respectively). Finally, leaf senescence started earlier in the full versus zero N treatment, though LAI trends of the two N treatments converged towards the end of the season.

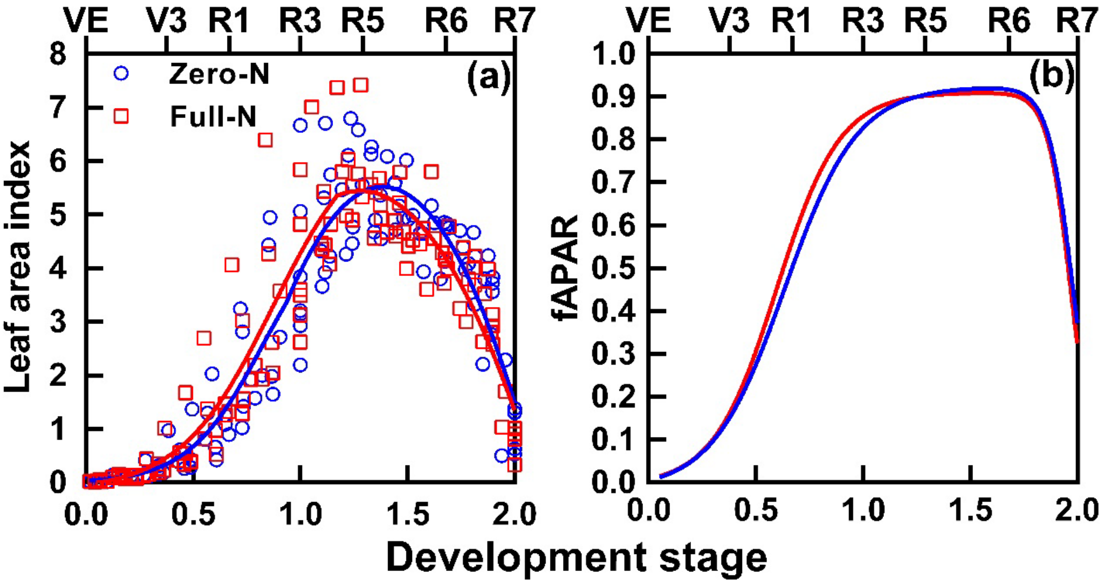

Figure 4-5. (a) Soybean leaf area index (LAI) and **(b)** estimated fraction of absorbed photosynthetically active radiation (fAPAR) in the full (red squares) and zero-N (blue circles) treatment as a function of developmental stage (DVS). Stages based on Fehr and Caviness (1977) are shown in the top x-axis. Solid lines represent the fitted models for

the full (red) and zero N treatment (blue) based on the pooled data across experiments. Data for each N treatment were pooled across environments.

Seasonal patterns in estimated fAPAR mimic the observed dynamics in LAI. The full N treatment exhibited consistently greater fAPAR between VE and R5, reaching 90% of full interception 4 d earlier compared with the zero N treatment (Figure 4-5b). These differences resulted in (2%) larger total estimated cumulative APAR from VE to R7 stages in the full versus zero N treatments (1410 versus 1383 MJ m^{-2}; p-value = 0.007). Differences in estimated APAR during the R3 to R6 phase between the full and zero N treatments (771 versus 766 MJ m^{-2}; p-value = 0.009) explained the greater accumulated ADM during this phase and resulting in greater seed number in the full N treatment.

Radiation-use efficiency was greater in the full versus zero N treatment in six of the seven environments (Figure 4-6). Overall, RUE was 8% greater in the full versus zero N treatment (2.00 versus 1.86 g m^{-2} MJ^{-1}, respectively). In contrast, we could not detect differences in leaf photosynthesis (i.e., net CO_2 assimilation rate) between N treatments (Figure 4-7). Crop stage influenced Amax and Ic ($p<0.04$), with Amax reaching a maximum (40.8 µmol CO_2 m^{-2} s^{-1}) around the R5 stage and Ic and Rd decreasing with ontogeny (Figure 4-6). However, none of the other photosynthesis parameters differed between N treatments ($p>0.1$), even though leaf N and specific leaf weight tended to be greater in the full versus zero N treatments (Supplementary Figure 4-S2).

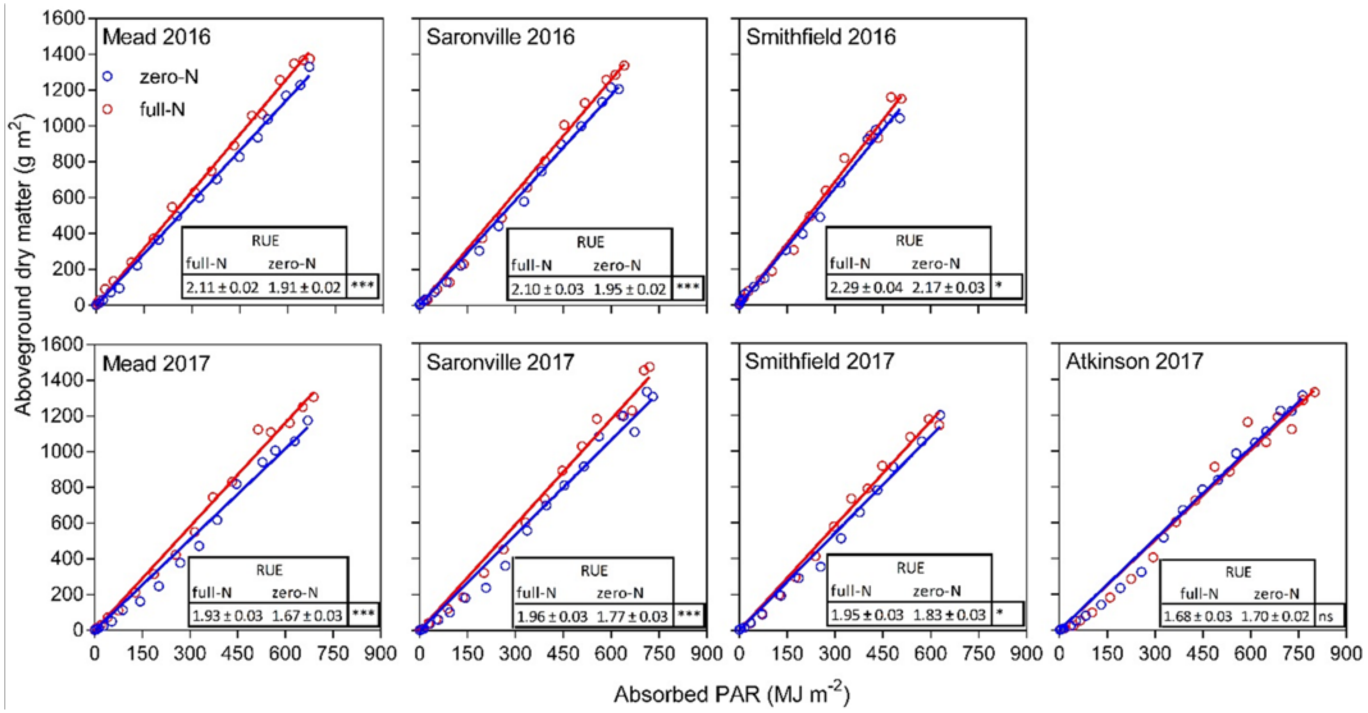

Figure 4-6. Aboveground biomass plotted against absorbed photosynthetically active radiation (APAR) in zero and full N treatments (blue and red symbols, respectively) across the seven environments. Slope of the fitted linear regression represents the radiation-use efficiency (RUE). Significance of the statistical test for the null hypothesis of no difference between slopes between N treatments is shown. Asterisks indicate significance a $p<0.10$(*), $P<0.05$ (**), or $P<0.001$ (***).

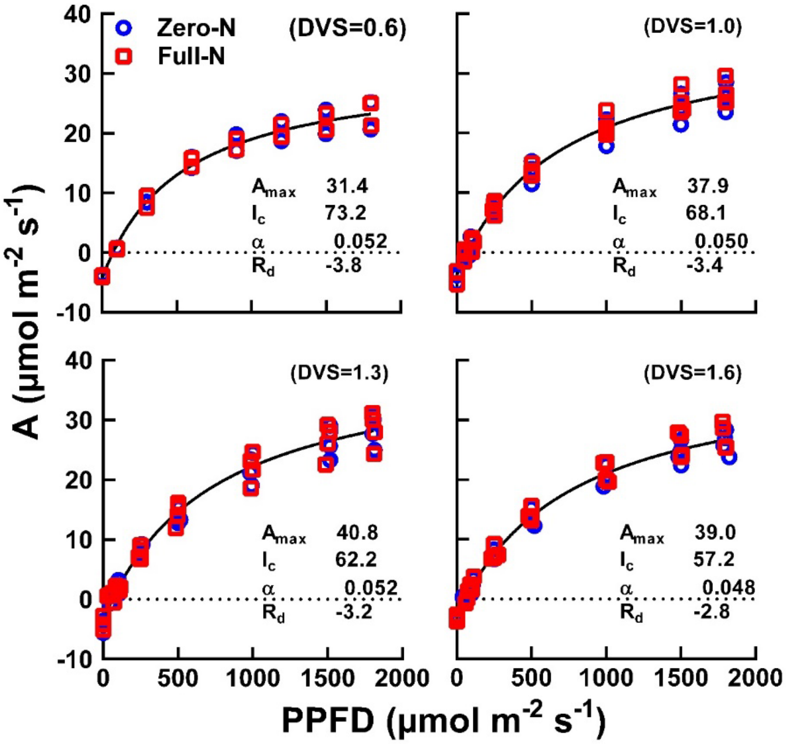

Figure 4-7. Leaf net photosynthesis (A) as a function of photosynthetic photon flux density (PPFD) in the full and zero-N treatment at four development stages (DVS). Parameters of the fitted models are shown: maximum photosynthesis in µmol CO_2 m^{-2} s^{-1} (Amax), light compensation point in µmol m^{-2} s^{-1} (Ic), initial slope of light response curve (α), and dark respiration in µmol CO2 m^{-2} s^{-1}(Rd). Data for each N treatment were pooled across environments.

4.3.5. Drivers for differences in seed weight and seed N between N treatments

Rate of accumulated seed dry matter during the seed filling period (R5-R7 phase) was greater in the full versus zero N treatment ($p = 0.032$). Considering that (i) both seed number and seed weight were consistently higher in the full versus zero N treatment (Table 4-2), yet (ii) seed filling duration (i.e., days between R5 and R7 stages) was identical between N treatments ($p = 0.356$), this result might indicate a higher rate of dry matter accumulation for individual seeds in the full versus zero N treatment. Unfortunately, we did not have detailed measurements of individual seed weight dynamics to confirm this hypothesis. Similarly, our weekly measurement of crop phenology may not have allowed us to detect small differences in seed filling duration between the N treatments. Still, the fact that we found that ample N supply resulted in both greater seed number and seed weight is remarkable considering the usual trade-off between seed number and individual seed weight.

There was a strong relationship between remobilized N from non-seed dry matter to seed and the amount of N in non-seed ADM at the R5 stage (Figure 4-9a). Remobilized N was 17% greater in the full versus zero N treatment (176 versus 150 kg N ha^{-1}, respectively, $p = 0.0005$; Table 4-1 and 2, Figure 4-8b). This difference in remobilized N was associated with greater accumulated N at R5 in the full versus zero N treatment (281versus 242 kg N ha^{-1}, respectively, $p<0.0001$), without changes in the fraction of non-seed N that was remobilized ($p=0.525$). On average, both treatments remobilized 62% of the N accumulated in the non-seed ADM at R5 (Figure 4-8a). These results indicate that the greater accumulated N before R5 in the full versus zero N treatment (Figure 4-3) translated into greater accumulated N at R5 which, given the stability in the fraction of N that is remobilized to seed from non-seed ADM, resulted in a greater absolute amount of remobilized N to the growing seeds (Figure 4-8). Apparent dry matter remobilization from non-seed ADM to seed was smaller than N remobilization (ca. 14% of non-seed ADM at R5), and did not differ among N treatments (Table 4-3; p = 0.283).

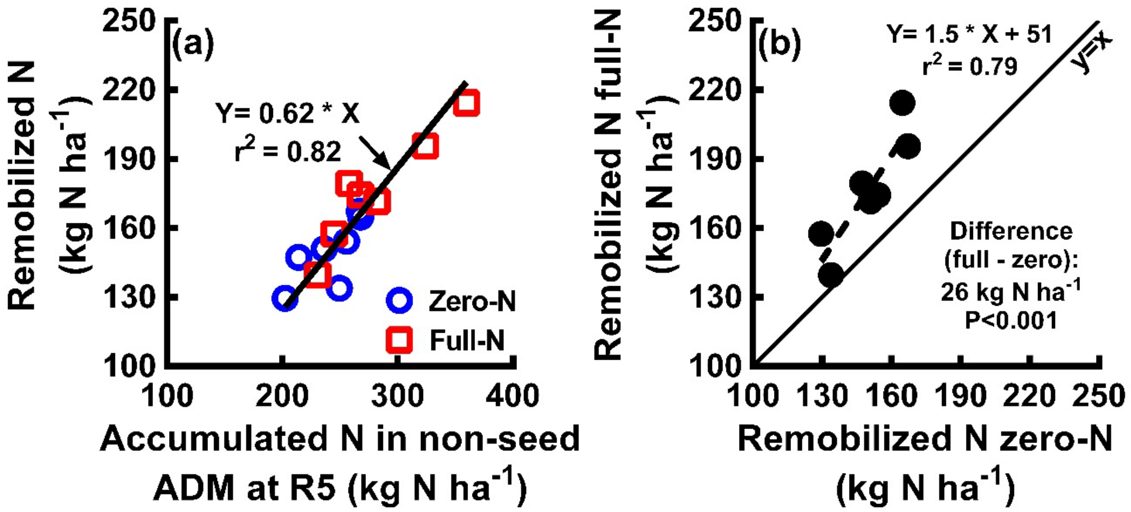

Figure 4-8. (a) Relationship between N remobilized from non-seed dry matter to seed during seed filling and the amount of accumulated N in non-seed dry matter at R5 in the full-N (red squares) and zero-N (blue circles) treatments. Parameters of the fitted linear regression (solid black line) and coefficient of determination (r^2) are shown. (b) Comparison of remobilized N between the full and zero-N treatments. Solid black line indicates y = x. Parameters of the fitted linear regression (dashed line) and coefficient of determination (r^2) are also shown. Treatment difference and significance are shown in (b).

4.4. Discussion

Differences in NAR between N treatments before the beginning of the seed fill indicate that N fixation and indigenous soil N supply did not meet crop N requirement in the zero N treatment. Higher NAR in the full versus zero N treatment supported faster leaf area expansion, greater APAR and CGR in the full N treatment, which allowed for greater seed set during the critical R3-R6 phase of development. Similarly, the extra N accumulated before R5 in the full versus zero N treatment (+45 kg N ha^{-1}) resulted in greater N remobilization from non-seed ADM to seed during the seed fill (+26 kg N ha^{-1}). This allowed the full N treatment to support greater mass per seed, and a higher seed N concentration compared with the zero N treatment, despite the former having greater seed number. This is consistent with Sinclair et al. (2003) and Sinclair and Rufty (2012), who postulated that the degree to which the plant can supply N to meet seed requirements will determine its capacity to meet the potential seed filling rate. These results suggest that strategies to increase N supply in soybean should aim to increase NAR before seed filling, with the goal of increasing the CGR during the critical period and increase the amount of N in non-seed ADM to support a greater seed weight and seed N concentration. Finally, leaf photosynthesis did not differ among N treatments, despite higher leaf N concentration in the full versus zero N treatment. This result was expected as leaf N concentration measured in the zero N treatments was above the level needed to maximize leaf photosynthesis as reported in previous studies (e.g., Sinclair and Horie, 1989). In contrast, canopy-level RUE was higher in the full versus zero N treatment, which could be associated with changes in root-to-shoot ratio and/or reduced N fixation costs due to lower N fixation as a result of N fertilizer application (Cassman et al., 1980; Pate and Layzell, 1990).

The degree to which seed N demand is met depends on (i) NAR during seed filling (R5-R7 stages of development), which includes N from soil and fixation, and (ii) N remobilized from non-seed ADM to the growing seeds (Stephenson and Wilson, 1977; Egli et al., 1985). In our study, NAR differed among N treatments during every stage of development, except seed filling (Tables 4-4 and 4-5). In contrast, , the quantity of N that was remobilized was greater in the full versus zero N treatment as a result of greater accumulated N in non-seed ADM at R5. These findings lead to two key inferences: (i) greater accumulated N in non-seed organs before R5 should not be seen as ‘luxury consumption’, as it helps sustain and even increase seed weight and seed N concentration; and (ii) in the full N treatment where soil N availability was assumed to be non-limiting, N remobilization (rather than higher NAR during seed fill) allowed for both greater seed mass and seed N concentration. These results are consistent with the assumptions that Boote et al. (1998) incorporated into the CropGro model, where the amount of remobilized N depends on plant development and is unaffected by N availability. In another study on fertilization of soybean with N, total N uptake and remobilization increased with fertilizer addition, whereas the contribution of N fixation to grain N declined (Kinugasa et al., 2012). This preference for remobilizing N from non-seed ADM may be associated with the lower cost of protein breakdown and re-synthesis compared with de novo protein synthesis (Penning de Vries et al., 1983). It may also indicate a loss of root functionality during seed fill as this has been reported for other crop species (Lisanti et al., 2013, Thibodeau and Jaworski 1975). Understanding the underlying drivers behind the apparent limited capacity to make use of available N in soil during seed fill deserves further research.

In a global context in which crop yields have to increase ca. 50% by year 2050 in order to meet food demand and avoid massive conversion of natural ecosystems into cropland (Cassman et al., 2003; van Ittersum et al., 2013), at issue is the degree to which N limitation may or not allow achievement of this goal in soybean. Given current average yield of ca. 3 Mg ha^{-1} in major producing areas, the goal would be to reach an average yield of 4.5 Mg ha^{-1} by year 2050, which has an associated N requirement of 360 kg N ha^{-1}. Indigenous soil N supply may be able to cover half of that N requirement considering that well-managed agricultural soils in USA and Argentina can provide ca. 100-150 kg N ha^{-1} (Cafaro La Menza et al., 2019). The rest of the N requirement (ca. 200-250 kg N ha^{-1}) may optimistically be supplied by fixation considering that it falls within the range of N fixation reported in the literature (see Salvagiotti et al., 2008). However, at issue is how to sustain yield gains in soybean production areas where average producer yields are already high, as it is the case for irrigated soybean in Nebraska and other areas in the Central US Great Plains (ca. 4.4 Mg ha^{-1}). In these environments, a 50%-yield increase goal would imply reaching an average yield of 6.8 Mg ha^{-1} by year 2050, which has an associated N requirement of 540 kg N ha^{-1}, which is similar to measured accumulated N in some of the full N crops in our study. Assuming the same level of indigenous soil N (150 kg N ha^{-1}), the N gap to be covered by fixation would then increase up to 400 kg N ha^{-1}. None of the recent reviews on N fixation on soybean provide evidence that combining that high level of N fixation and indigenous soil N supply is possible (Salvagiotti et al., 2008; Ciampitti and Salvagiotti, 2018). While our N fertilizer treatment was successful at increasing both yield and grain N concentration, it was far from being cost-effective and obviously not environmentally sound to be adopted by commercial farms. Increasing N fixation potential and/or indigenous soil N supply are avenues worth exploring to overcome the N limitation in soybean though the available room to improve these parameters, and associated timeline and costs, are unknown.

4.5. References

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4.6. Supplementary material

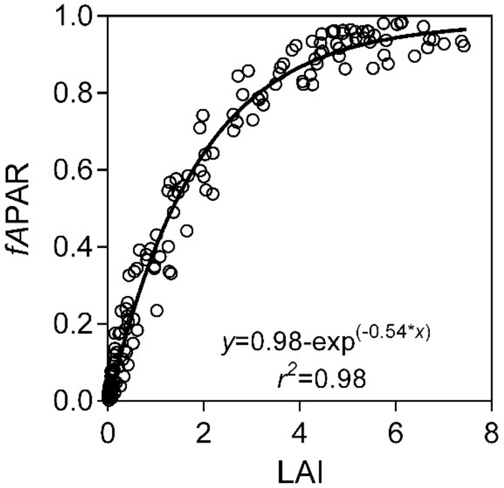

Figure 4-S1. Daily fraction of absorbed photosynthetically active radiation by green leaf area (fAPAR) as a function of green leaf area index (LAI).

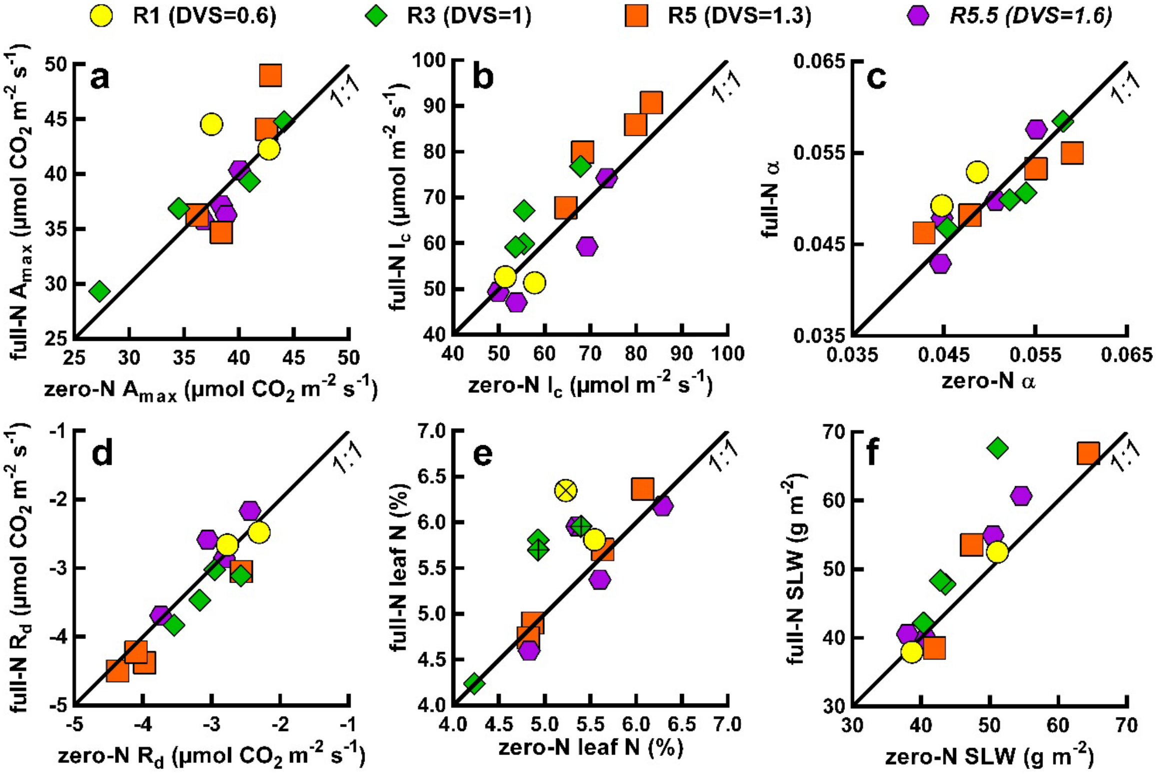

Figure 4-S2. Comparison of zero versus full N treatment in **(a)** maximum photosynthetic rate at light saturation (A_{max}), **(b)** light compensation point (I_c), **(c)** initial slope of the light response curve (α), **(d)** dark respiration (Rd), **(e)** leaf N, and **(f)** specific leaf weight (SLW). Solid diagonal black line indicates the 1:1 line. Symbols and colors indicate the development stage (DVS) at which assessments were made. Crosses inside symbols indicate cases with statistically significant differences between N treatments (p value < 0.05)

CHAPTER 5: ASYNCHRONY OF BIOLOGICAL NITROGEN FIXATION AND CROP NITROGEN DEMAND IN HIGH-YIELD SOYBEAN

This chapter will be submitted to Field Crop Research journal: Nicolas Cafaro La Menza, Murray Unkovich, Michael Castellano, Juan P. Monzon, James E. Specht, Timothy J. Arkebauer, John Lindquist, Johannes M. H. Knops, Patricio Grassini (2019). Asynchrony of biological nitrogen fixation and crop nitrogen demand in high-yield soybean.

Abstract

Indigenous soil N supply (ISN) and biological N_2 fixation (BNF) are not sufficient to fulfill plant N demand in high-yield environments. The causes explaining why the combined N supply from fixation and ISN is not able to meet plant N demand are unknown. This is critical as average soybean yields get closer to their yield potential and, hence, the N limitation is likely to become larger. The objective of the study was to examine seasonal patterns in BNF and ISN to understand why they are not sufficient to fulfill plant N demand in high-yield soybean crops. We conducted five experiments in high-yield production environments in Nebraska, USA (yield range: 5.3 to 6.7 Mg ha^{-1}). Each environment included a treatment that relied upon ISN and BNF (‘zero N’) and a full N treatment that received ample N supply through successive N fertilizer applications (N demand). The full N season was used to establish the plant N demand at a given point of time. Differences in accumulated N between the full and zero N treatments were used to calculate the N limitation (‘N gap’). Sigmoidal models were used to portray seasonal dynamics of N demand, BNF, and ISN. Relationships between the N gap and seasonal patterns of BNF, ISN, and plant N demand were explored. There was a trade-off between BNF and ISN with BNF reduced less than proportional per unit increase in ISN. There was a temporal asynchrony between BNF and N demand, that is, BNF was not sufficient to meet plant N demand as the latter increased and the contribution of ISN decreased. Indeed, peak in plant N demand occurred after the peak in ISN but before the peak in BNF. The peak of ISN supply was the most important factor explaining variation in the N gap across environments. Results from this study can help develop management practices aiming to reduce soybean N limitation in high-yield environments.

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Keywords: soybean, Glycine max (L.) Merr., nitrogen fixation, nitrogen demand, indigenous soil nitrogen supply.

5.1. Introduction

Soybean [*Glycine max* (L.) Merr.] has a large nitrogen (N) requirement per unit of seed yield produced (Sinclair and de Wit, 1975). The main sources of N in soybean are biological N_2 fixation (BNF) and indigenous soil N supply (ISN), which includes soil organic matter mineralization, dry and wet atmospheric deposition, N from irrigation water, and a small amount of N fertilizer sometimes applied as ‘starter’ at sowing. Ideally, as yield increases and ISN falls short to meet plant N demand, crops would rely more and more on the contribution from BNF (Giller and Cadisch, 1995). However, recent studies found that, even in optimal crop growing conditions, BNF is not sufficient to fulfill plant N demand, especially in high-yield environments with low ISN, leading to a reduction in seed yield and seed protein concentration (Cafaro La Menza et al., 2017; 2019a). The causes underlying the apparent inability of BNF to meet seasonal plant N demand in high-yield soybean have not been determined. This is critical as average soybean yields get closer to their yield potential due to genetic and agronomic improvements and, hence, increasing the odds of N limitation.

Ranges of BNF at physiological maturity are summarized in recent review articles (Salvagiotti et al., 2008; Ciampitti and Salvagiotti, 2018) and some studies have also described seasonal BNF dynamic for soybean (Salvagiotti et al., 2009; Zapata et al., 1987; Cordova et al., 2019). However, we are not aware of studies assessing the contribution of BNF and ISN in relation with the plant N demand during the cropping season. Such an assessment would require a comparison of ISN and BNF dynamics in a crop that relies exclusively on N from BNF and ISN (zero N crop) *versus* a crop that received ample N supply to ensure no N limitation (full N crop). This evaluation would also need to be conducted in high-yield environments where the N limitation is most likely to occur (Cafaro La Menza et al., 2017) and would require that yield-limiting and reducing factors (besides N in the zero N treatment) are effectively controlled to ensure the crop grows in near-optimal conditions.

Previous research showed that most (ca. 80%) of the accumulated N at physiological maturity (R7)[2] is absorbed during the reproductive stages (Thies et al., 1995; Bender et al., 2015), with maximum N accumulation rates occurring during pod setting, that is, between R3 and R5 (Gaspar et al., 2017). In contrast, maximum rates of BNF occurs around R5 (Zapata et al., 1987; Mastrodomenico and Purcell, 2012; Cordova el al., 2019). This findings indicates that there is an apparent asynchrony between the time of occurrence of maximum BNF and maximum accumulated N rates. Besides this asynchrony, ISN may also influence the patterns of BNF during the growing season considering the well-known trade-off between BNF and ISN (Streeter and Wong, 1988; Salvagiotti et al., 2008; Santachiara et al., 2017). There has been no explicit evaluation on how the asynchrony between BNF and N demand, together with ISN, may explain the N limitation in high-yield soybean.

There is clearly a knowledge gap in relation to the factors influencing the capacity of BNF to meet plant N demand of high-yield soybean. The objective of the present study was to determine the causes explaining the apparent inability of seasonal contribution of BNF and ISN to fulfill plant N demand in high-yield soybean crops. We assessed seasonal dynamics of N demand, ISN, and BNF in irrigated experiments conducted in

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[2] In this report, we used the phenological stages defined by Fehr and Caviness (1977). V2: fully developed trifoliolate leaf at node above the unifoliolate node; V4: four nodes on the main stem with fully developed leaves beginning with the unifoliolate node; R1: (beginning of bloom) one open flower at any node on the main stem; R3: (beginning of pod setting) pod of 5 mm long at one of the four uppermost nodes on the main stem with a fully developed leaf; R5: (beginning of seed filling) a seed 3 mm long in a pod at one of the four uppermost nodes on the main stem with a fully developed leaf; R7: physiological maturity.

high-yield production environments in Nebraska, US (yield range: 5.3 to 6.7 Mg ha^{-1}). This study extends the findings from previous studies in soybean looking into the physiological drivers for the N limitation by looking into BNF and ISN dynamics during the entire growing season (Cafaro La Menza et al., 2017, 2019a).

5.3. Materials and methods

5.3.1. Experimental sites and design

The set of high-yield experiments conducted in Nebraska (NE), USA reported by Cafaro La Menza et al. (2017, 2019a) were used in this study. The irrigated production environment of NE provides a suitable background to evaluate the N limitation in soybean. Experiments were conducted in pivot-irrigated producer fields located at three sites in NE (Atkinson, Mead, Saronville, and Smithfield) during two crop seasons (2016 and 2017). These fields achieved high soybean yields in previous years (>5 Mg ha^{-1}) and portrayed well the range of weather, soils, and management practices (e.g., tillage methods, cultivar maturity groups [MGs]) across NE. In this study, we excluded the two years of experiments at Atkinson because of the high NO_3^- concentration (ca. 20 ppm) in the irrigation water, which, together with a large irrigation amount applied in his field, made estimation of BNF impossible following the natural abundance of ^{15}N technique. Similarly, the experiment conducted at Saronville in 2017 was excluded because the determinations of soil ^{15}N and soybean ^{15}N concentration followed unrealistic patterns.

So, the final database consisted of five out of the seven environments from Cafaro La Menza et al. (2017, 2019a).

Each experiment included two N treatments: (i) a 'zero N' treatment in which the crop relied on ISN and BNF, and (ii) a 'full N' treatment especially designed to provide the crop with an ample N supply (by applying fertilizer N) to optimally match seasonal crop N demand (see Section 5.2.2). Treatments were allocated in a complete randomized design with four replicates per treatment (size: 176 m^2 each). Experimental plots were purposely placed in areas within each field where highest (maize and/or soybean) yields were achieved in previous years. Soybean was always grown in a 2-y rotation with maize, which is the dominant practice in the US Corn belt (Grassini et al., 2014). Soils were deep, without physical or chemical constrains to root growth. Soil series were Yutan silty clay loam (Mead), Hastings silt loam (Saronville), and Holdrege silt loam (Smithfield). Crops were grown with the explicit goal of removing any yield-limiting factor (except for N in the zero-N treatment). In all cases, seeds were treated with fungicide and insecticide, but received no inoculant as lack of inoculation is a common management practice for soybean-maize rotation fields in the US Corn Belt, and there is typically a lack of yield response to inoculation in high-yield environments (de Bruin et al., 2010; Leggett et al., 2017 and references cited therein). Row spacing was 0.76 m in all environments, with seeding rates well above the recommended plant density to maximize soybean yields (De Bruin and Pederson, 2009). Soil water content in the upper meter was monitored using Watermark® sensors and maintained above 65% of available water throughout the entire growing season. Several prophylactic foliar applications of herbicide, fungicide, and insecticide, and pre-sowing nutrient applications (based on soil test results) kept the crops free from biotic and nutrient stresses (besides N in the zero N treatment). Soil samples were collected at sowing to determine soil texture (clay, silt, and sand fractions), $N\text{-}NO_3^-$ concentration, and soil organic matter. In each experiment, four soil cores were collected from three of the four replicates, split into 0−0.3, 0.3−0.6, 0.6−0.9, and 0.9–1.2 m segments, and combined into one composite sample per depth. Soil $N\text{-}NO_3^-$ at sowing ranged from 32 to 58 kg of N ha^{-1} (0-0.6 m depth) and from 43 to 94 kg of N ha^{-1} (0-1.2 m depth) across environments. Daily weather data (solar radiation, maximum and minimum temperature, precipitation, relative humidity, and wind speed) were collected from a portable weather station located adjacent to the experiments. Detailed description of the experiments is provided elsewhere (Cafaro La Menza et al., 2017, 2019a, b).

5.3.2. Nitrogen treatments

As mentioned previously, each experiment included a zero N treatment that relied on BNF and ISN, and a full N treatment that received ample N supply. In the case of the full N treatment, a total amount of 870 kg N ha^{-1} was applied in all sites to fully satisfy the plant N demand and maximize yield. The N fertilizer was applied as urea and broadcast between plant rows in the full-N treatment. This amount was calculated based on (i) site-specific yield potential simulated using the SoySim model (Setiyono et al., 2010), (ii) N uptake requirement of 80 kg N per Mg^{-1} seed yield (Salvagiotti et al., 2008; Tamagno et al., 2017), and (iii) fertilizer recovery efficiency of 70% to account for unavoidable N losses. Total N fertilizer amount was split into five successive applications (of increasingly higher amounts) to optimally match the expected increase in crop N requirement during the crop season (Thies et al., 1995; Bender et al., 2015). In our experiments, 10%, 10%, 20%, 30%, and 30% of the total N fertilizer was applied at V2, V4, R1, R3, and R5, respectively. To summarize, the full N treatment was explicitly designed to preclude any N limitation by supplementing with fertilizer N any site-specific insufficiency of N supply during the growing season. Also, the N protocol helped avoid the confounding effect involving the trade-off between BNF and ISN by providing enough N to meet plant N demand irrespective of the contribution from BNF and ISN.

An N-omission maize plot (used as reference crop for BNF determination) was sown adjacent (<30 m) to the soybean experiment in each environment. In each environment, the maize plot (9.1 x 11 m and 3.8 x 10 m) was sown on the same date as soybean and did not receive any N fertilizer (but received P and K fertilizer in similar amounts to the surrounding soybean crop). Logistical issues did not allow us to have replicates of the maize plot. Final stand count was 9 maize plants m^{-2}. Plots received irrigation to avoid water limitation and were kept free of weeds, diseases, and pathogens during the entire crop season. The maize hybrid (DKC61-54RIB) was selected to portray modern high-yield genetics with broad adaptability and good pest resistance and having similar growth duration to the soybean variety planted in each environment.

5.3.3. Field measurements

The five experiments included detailed measurements of phenology, and accumulated ADM and N during the entire crop season. We track crop phenology on a weekly basis following Fehr and Caviness (1977) staging system by recording the crop stage in ten consecutive plants within one row in each replicate for all treatments and environments, starting at VE and concluding at R7. Seasonal dynamics of N uptake were assessed in each environment and treatment by collecting a 1-m row of consecutive plants (same sampling area as for the phenology assessment), surrounded by two rows (0.76 m between rows) receiving the same N treatment, in each replicate. We did not attempt to collect root biomass. Samples were collected weekly from VE until R7 and separated into different plant organs (green leaves, stems, seed, pod walls, and senesced leaves). Abscised leaves were also collected every week from a 1 m row net placed in-between rows in each replicate. Plant tissue samples were oven-dried at 70 °C until reaching constant weight. Each plant tissue sample was separately ground in a Wiley mill (1-mm screen mesh), and N concentration was determined with a dry combustion-based analyzer (LECO Corporation, St Joseph, MI). Accumulated N in ADM was calculated based on the ADM and N concentration of each plant tissue and the sum of all plant components. Similarly, larger plant samples (4.6 m^2) were collected shortly after R7 from the two central rows in each plot, surrounded by two rows receiving the same N treatment, to obtain an end-of-season estimate of seed yield. Seed yield and seed weight were adjusted to 0.130 kg H_2O kg^{-1} seed, which is the standard moisture content in soybean.

Seasonal dynamics of BNF and ISN were determined using the natural ^{15}N abundance method (Shearer and Kohl, 1986). We only determined BNF and ISN for the zero N treatment; the natural abundance method was not applicable to the full N treatment due to the expected isotopic fractionation caused by increases in denitrification as a result of N fertilizer addition (Mathieu et al., 2007). Every week, we collected aboveground plant samples from (i) 0.5-m row of consecutive soybean plants in the zero-N (next to the 1-m row of accumulated ADM sampling), and (ii) a two maize plants in the N-omission (to be used as ‘reference’ for ^{15}N determination). These samples were collected at the same time as for phenology, ADM, and accumulated N samples and, similarly, were oven-dried and ground to determine ^{15}N abundance using an automated continuous-flow isotope-ratio mass spectrometer (Finnigan MAT, Bremen, Germany). The proportion of fixed N was calculated as:

$$BNF\ (\%) = 100 * \frac{\delta 15\mathrm{Nref} - \delta 15\mathrm{Nsoy}}{\delta 15\mathrm{Nref} - \mathrm{B}} \qquad \text{Eq (1)}$$

where δ^{15}Nref and δ^{15}Nsoy are the natural ^{15}N abundance of the reference crop (maize from the N-omission plot) and soybean, respectively, and *B* is the ^{15}N natural abundance of N in soybean that relies only on BNF. The B value used in this work was -1.75, which corresponds to the average value reported in the literature as suggested by Unkovich et al. (2008). In the case of δ^{15}Nref, we adjusted a quadratic model to smooth the observed variation among sampling times, with r^2 of fitted models ranging from 0.67 to 0.93 across experiments. BNF for a given sampling time was calculated based on the percentage of

BNF and accumulated N. ISN was calculated as the difference between accumulated N and BNF.

5.3.4. Data analysis

Following previous studies (e.g., Lindquist et al., 2005), crop development was made comparable across experiments (with different temperatures, sowing dates, and cultivar MG) by defining developmental stage (DVS) using a dimensionless scale where 0, 1, and 2 correspond to VE, R3, and R7 stages, respectively. In our case, developmental rate was calculated based on mean air temperature using a beta function as defined by Wang and Engel (1998), with appropriate cardinal temperatures for each phase as reported by Setiyono et al. (2007). In our scale, DVS values of 0.5 and 1.5 would mean that half of the thermal units between VE-R3 and R3-R7, respectively, have been accumulated. We did not account for photoperiod on our DVS calculation as the four locations were located within a narrow latitudinal band (from 40.5º to 42.6º).

We used accumulated N in the full N treatment as an estimate of the plant N demand over time. In the case of the zero N treatment, we used accumulated N to determine the overall contribution from BNF and ISN. A sigmoidal model was fitted to seasonal dynamics of accumulated N (both full and zero N treatments), BNF and ISN (only in zero N treatment) *versus* DVS and subsequently used to derive the daily N demand, BNF, and ISN rates. Comparison of N accumulation rates in the full *versus* zero N treatment would provide an indication of the degree of N limitation (hereafter called ‘N gap’) at a given point of time. The first derivative of the sigmoidal models relating plant N demand (in the full N) or BNF and ISN (zero N) with DVS were used to derive the following parameters: maximum rates of BNF (BNF_{MAX}), N demand (DEM_{MAX}), and ISN (ISN_{MAX}) and their respective time of occurrence ($TBNF_{MAX,}$ $TDEM_{MAX,}$ and $TISN_{MAX}$). The second derivative was used to derive the approximate time when BNF started to actively fix N ($TFIX_1$) and ceased ($TFIX_2$) to be a major source of N supply and the duration of the phase encompassed between these two times (DFIX) (Figure 5-1).

A three-segment linear model was adjusted to seasonal dynamics in accumulated N gap (i.e., difference between accumulated N in full versus zero N treatments) in each experiment to identify the specific crop phases when the overall N supply from BNF and ISN was not sufficient to fulfill plan N demand (Figure 5-1). Briefly, the three-segment model consists of a first phase where an incipient N gap developed, followed by a second phase where the N gap increases substantially until reaching a plateau, indicating the start of the third phase. From the three-phase linear model of each experiment, we derived the following parameters: rate at which the N limitation increases during phase I (NR_1) and phase II (NR_2), duration of phase I (ND_1) and phase II (ND_2), and N gap (CNgap).

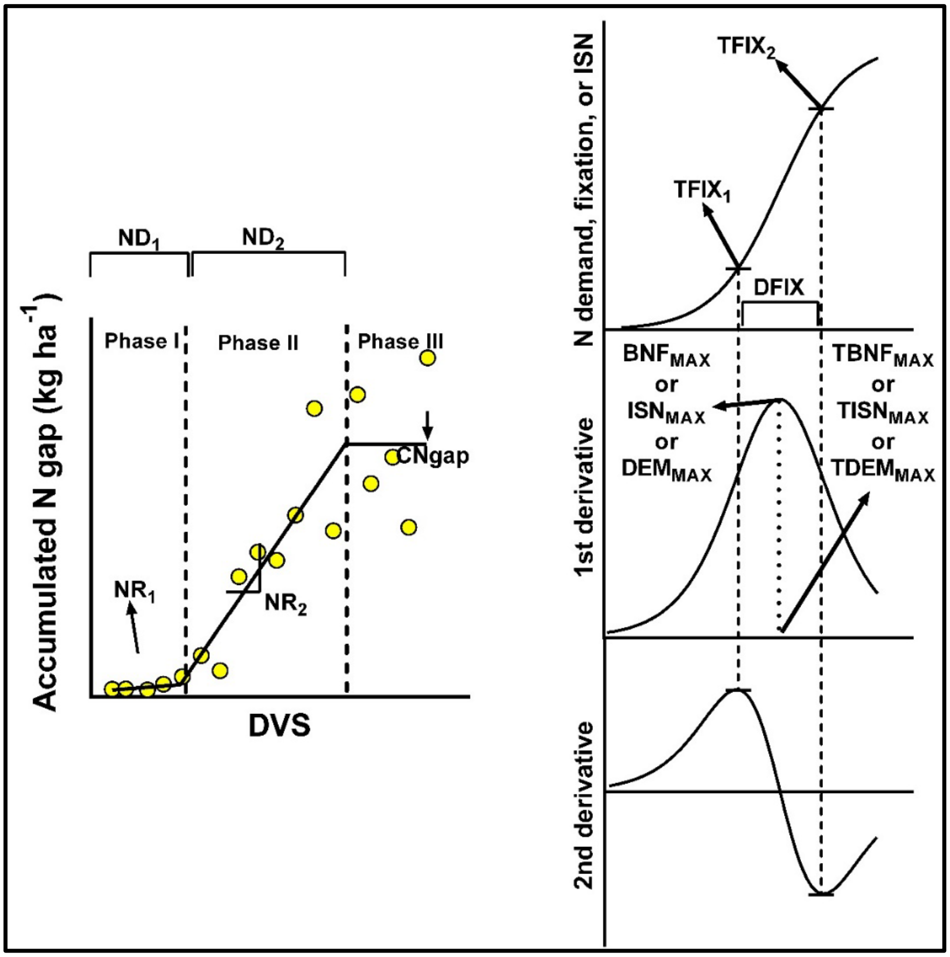

Figure 5-1. Scheme showing the fitted three-segment linear (left) and sigmoidal models (right) to describe seasonal patterns on accumulated N gap, BNF, and ISN. Parameters of the three-phase linear models are: N gap rate during phase I (NR_1) and phase II (NR_2), duration of phase I (ND_1) and phase II (ND_2), and cumulative N gap of phase III (CNgap). First and second derivatives were used to derive a number of parameters from the fitted sigmoidal models: maximum N demand (DEM_{MAX}), BNF (BNF_{MAX}), ISN (ISN_{MAX}) rates and their time of occurrence ($TBNF_{MAX}$, $TDEM_{MAX}$, $TISN_{MAX}$), the time

when BNF started to actively fix N ($TFIX_1$) and ceased ($TFIX_2$), and duration of this phase (DFIX).

Each site-year combination (total of five) is referred hereafter to as an ‘environment’. We used a combined analysis of variance (ANOVA) to analyze the effect of N treatments on seed yield and accumulated N across environments (SAS® PROC MIXED v.9.3; Moore and Dixon, 2015). The combined ANOVA assumes homogeneity within and between environment’s variances. Indeed, variances were not too different ($Fmax < 6$); therefore, our analysis can be considered robust (Milliken and Johnson, 2009). Both environments and N treatments were treated as fixed effects. In this analysis, environments cannot be directly compared, but the analysis can provide an estimate of the overall significance and consistency of the differences between N treatments across environments.

As a first step, Pearson’s correlation analysis was used to investigate relationships between N gap and the parameters of the three-phase linear models (ND_1, ND_2, NR_1, and NR_2) (InfoStat; Di Rienzo et al., 2011). Subsequently, we used Person’s correlation analysis to explore associations between (i) Ngap and the parameters derived from the seasonal patterns of BNF, ISN, and N demand (BNF_{MAX}, DEM_{MAX}, ISN_{MAX}, $TBNF_{MAX}$, $TDEM_{MAX}$, $TISN_{MAX}$, $TFIX_1$, $TFIX_2$, DFIX) and (ii) parameters of the three-phase linear models (ND_1, ND_2, NR_1, and NR_2) and those derived from the seasonal patterns of BNF, ISN, and N demand. The goal of these analyses was to understand which of these parameters explained variation in N gap across environments.

5.4. Results

5.4.1. Seed yield, accumulated N, BNF, and ISN

Soybean seed yield ranged from 5.3 to 6.7 Mg ha^{-1} across treatments and environments. In the zero N treatment, ISN and BNF averaged 128 and 257 kg N ha^{-1}, respectively, with the latter ranging from 218 to 293 kg N ha^{-1} across environments (Table 5-1, Fig. 5.2). On average, BNF represented 67% of accumulated N at R7 (range: 59 to 80%). Despite the large amount of fixed N, the accumulated N gap at R7 (calculated as the difference in accumulated N in the full *versus* full N) averaged 47 kg of N ha^{-1} across environments (range: 20 to 89 kg N ha^{-1}). Consistent with the observation of N gap, seed yield was 11% higher in full *versus* zero N treatment (6.1 *versus* 5.5 Mg ha^{-1}, respectively). To summarize, differences in seed yield and N gap in zero *versus* full N treatment indicated that BNF and ISN were not sufficient to meet plant N demand. Finally, there was a weak (but still statistically significant) negative correlation between BNF and ISN, which was consistent with the notion of a trade-off between these parameters (Fig. 5-2). However, the slope derived from the relationship (-0.56 kg N fixed per kg ISN) indicates that the reduction in BNF per additional unit of ISN was less than proportional.

Table 5-1. Mean soybean seed yield, accumulated nitrogen (N) at R7, biological N_2 fixation and its contribution to total N uptake, and indigenous soil N supply (ISN).

Location	Year	Treatment	Seed yield (Mg ha^{-1})	Accumulated N at R7 (kg N ha^{-1})	Biological N_2 fixation at R7 (kg N ha^{-1})	N_2 fixation (%)	ISN (kg N ha^{-1})
Mead	2016	zero	5.3	419	264	63	155
		full	6.2	460	-	-	-
	2017	zero	5.4	370	242	65	128
		full	6.7	459	-	-	-
Saronville	2016	zero	5.8	408	270	66	138
		full	6.4	436	-	-	-
Smithfield	2016	zero	5.3	364	293	80	71
		full	5.7	384	-	-	-
	2017	zero	5.5	367	218	59	149
		full	5.5	432	-	-	-

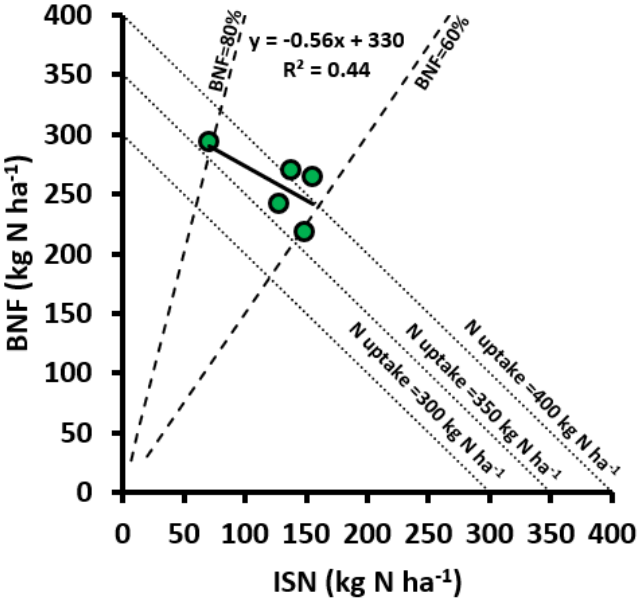

Figure 5-2. Trade-off between biological N2 fixation (BNF) and indigenous soil N supply (ISN) measured at physiological maturity (R7). Range of relative contribution of BNF are indicated with dashed lines. Dotted isolines indicate different levels of accumulated N at R7. The general framework has been adapted from that used by Santachiara et al. (2017).

5.4.2. Dynamics of nitrogen demand, BNF, ISN, and accumulated Ngap

Seasonal dynamics in plant N demand, BNF, and ISN followed a sigmoidal pattern (Figure 5-3 left). Accumulated BNF and ISN represented 8% and 34% (R3 stage) and 25% and 80% (R5 stage) of the accumulated BNF and ISN at R7 in the zero N treatment. Plant N demand and BNF increased gradually, reaching maximum rates at different stages (DVS = 1.28 and 1.54, respectively). While plant N demand peaked around R5 stage, maximum rate of BNF occurred ca. R5.5 stage. Both plant N demand and BNF declined during the seed filling, with BNF accounting for 85% of accumulated N after R5. In contrast, highest of ISN rates were observed ca. R3 stage, declining sharply afterwards (Figure 5-3 right). Contribution of ISN to the N accumulated during the seed filling was 15%.

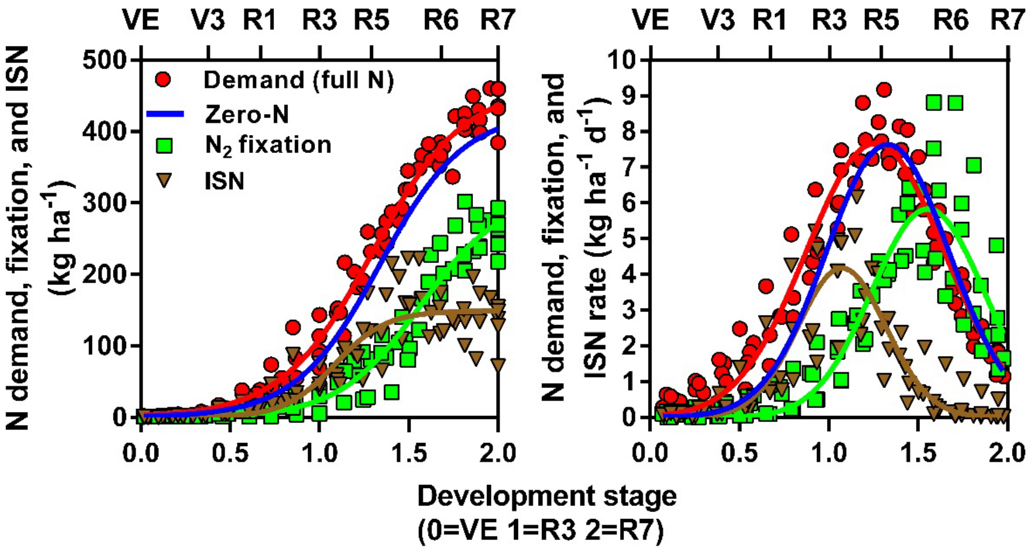

Figure 5-3: (Left) Dynamics of nitrogen (N) plant demand, biological N_2 fixation (BNF), indigenous soil N supply (ISN), and combined supply from BNF and ISN (zero N) from emergence (VE) to physiological maturity (R7) in five high-yield experiments conducted in Nebraska, US. Respective rates are shown in the right panels.

The overall N supply from ISN and fixation was not sufficient to meet plant N demand before R5 stage, generating an N gap. Dynamics in accumulated N gap were described using a three-segment model (Figure 5-4). The first phase (phase I) started at VE and ended shortly before R1 with a relatively small accumulated N gap (< 3 kg N ha^{-1}). The second phase (phase II) started ca. R1 and concluded shortly before R5; this phase represented the period of the most severe N limitation. Accumulated N gap increased linearly with developmental time during phase II until ca. R5. The last phase (phase III) started at R5 and finished at R7. The accumulated N gap remained unchanged during phase III, indicating that there was no N limitation; hence, the accumulated N gap at R7 was almost identical to the accumulated N gap by R5 (Fig. 5-4). To summarize, most of the differences in accumulated N gap were explained by differences in accumulated N during phase II as a result of insufficient N supply from ISN and BNF to meet plant N demand.

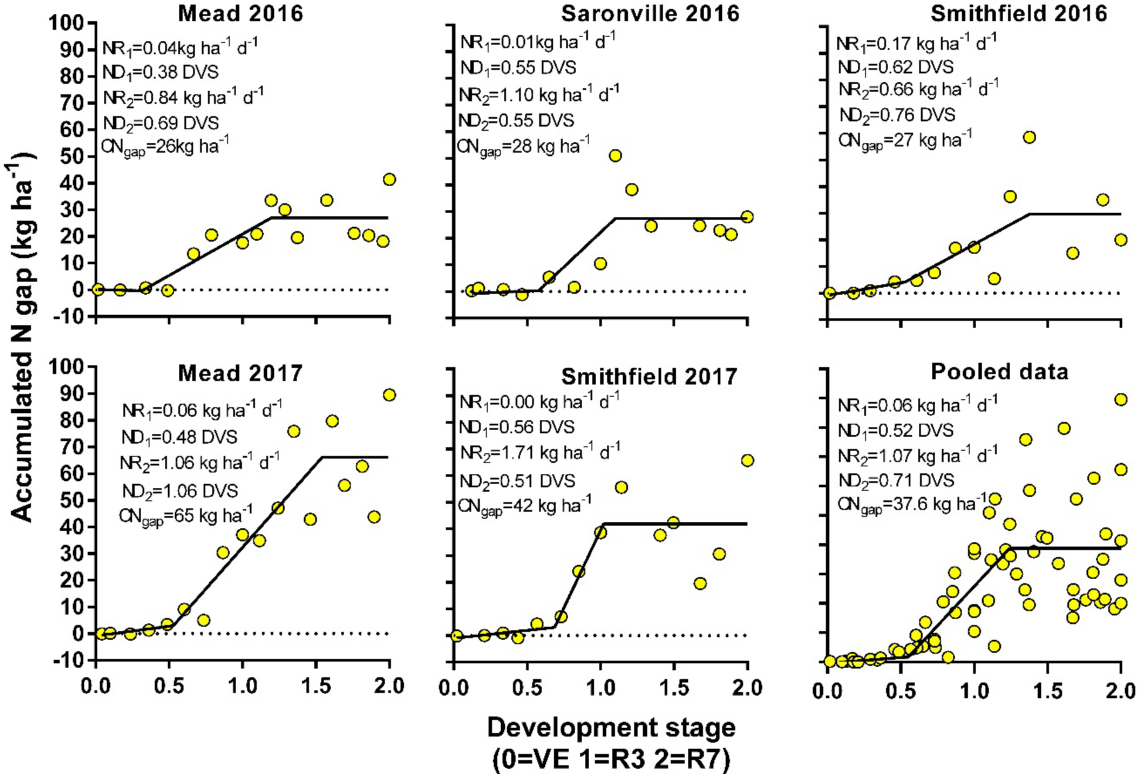

Figure 5-4. Accumulated N gap from emergence (VE) to physiological maturity (R7) in five high-yield environments.

5.4.3. Linkage of accumulated N gap to different aspects of BNF, ISN, and N demand across environments

Accumulated N gap at R7 was not correlated with any of the four parameters describing the three-phase seasonal accumulated N gap pattern (Table 5-2). In other words, across environments, the accumulated N gap resulted from different combinations of rates and duration of the two phases. From all the parameters derived from the seasonal dynamics of ISN, BNF and N demand, only ISN_{MAX} explained a substantially portion of the observed variation in N gap environments ($r = -0.89$; $p<0.05$).

Table 5-2. Pearson correlation coefficient (r) for the relationships between accumulated N gap at R7 (CN gap) *versus* parameters describing the seasonal accumulated N gap (NR1, NR2, ND1, ND2), BNF (BNF_{MAX}, $TBNF_{MAX}$, $TFIX_1$, $TFIX_2$, DFIX), ISN (ISN_{MAX}, $TISN_{MAX}$) and N demand patterns (DEM_{MAX}, $TDEM_{MAX}$).

	r	*p*-value
Parameters from N gap pattern:		
NR_1	-0.16	0.793
NR_2	0.35	0.563
ND_1	-0.11	0.866
ND_2	0.68	0.203
Parameters from BNF pattern:		
BNF_{MAX}	-0.46	0.438
$TBNF_{MAX}$	-0.72	0.174
$TFIX_1$	-0.63	0.253
$TFIX_2$	-0.85	0.072
DFIX	0.42	0.484
Parameters from ISN pattern:		
ISN_{MAX}	-0.89	0.046
$TISN_{MAX}$	0.51	0.377
Parameters of N demand:		
DEM_{MAX}	-0.38	0.529
$TDEM_{MAX}$	-0.16	0.800

Rate at which the N limitation increases during phase I (NR1) and in phase II (NR2), duration of phase I (ND1) and phase II (ND2), and cumulative N gap of phase III (CNgap). Maximum N demand, BNF, and ISN rates and their time of occurrence (BNF_{MAX}, DEM_{MAX}, ISN_{MAX}, $TBNF_{MAX}$, $TDEM_{MAX}$, $TISN_{MAX}$), the time when BNF started to actively fix N ($TFIX_1$) and ceased ($TFIX_2$), and duration of this phase (DFIX).

It was also possible to explore some environmental drives explaining differences in the parameters associated with the accumulated N gap. For example, there was a strong negative correlation between the ND_2 and $ISN_{MAX,}$ and a positive correlation between ND_1 and BNF_{MAX} (Table 5-3). On the one hand, some of these associations were expected, for example, increasing ISN leading to a shorter duration of the phase 2. On the other hand, some other relationships were difficult to interpret, for example, between maximum BNF rates and duration of phase 1. Besides these associations, we also found strong correlations between ISN_{MAX} and soil N-NO_3^- at sowing in the upper 1.2 m (r^2=0.91; p=0.03).

Table 5-3. Pearson correlation coefficients of correlation between accumulated N gap parameters and the parameters describing dynamics of BNF, ISN, and N demand.

	NR_1	NR_2	ND_1	ND_2
Parameters of N_2 fixation:				
BNF_{MAX}	0.51	nil	0.88*	-0.45
$TBNF_{MAX}$	0.36	nil	0.65	-0.67
$TFIX_1$	0.39	-0.04	0.7	-0.63
$TFIX_2$	0.56	-0.42	0.56	-0.49
DFIX	-0.23	-0.31	-0.69	0.62
Parameters of ISN:				
ISN_{MAX}	-0.14	0.07	0.30	-0.94*
$TISN_{MAX}$	-0.10	-0.35	-0.55	0.74

Parameters of N demand:				
DEM_{MAX}	0.78	-0.43	0.83	-0.06
$TDEM_{MAX}$	0.19	-0.73	-0.29	0.37

Significance at *p<0.05, **p <0.01, ***p<0.001

5.5. Discussion

We are not aware of previous studies assessing patterns in BNF and ISN during the entire crop season in soybean production environments where yield potential (as estimated from our full N treatment) ranged from 5.5 to 6.7 Mg ha^{-1}. Similarly, our study was the first to compare seasonal patterns of BNF and ISN with plant N demand estimated from a treatment where soybean crops received ample N supply. The dynamics of BNF in this study were similar to previous reports (Cordova et al., 2019) but maximum N fixation rates were notably higher (ca. 6 *versus* 3 kg N ha d^{-1}). Indeed, range of BNF in our study (218-293 kg N ha^{-1}) was near the maximum values of N fixation reported in the literature of ca. 300 kg N ha^{-1} (Ciampitti and Salvagiotti, 2018). The higher N fixation rate (6 kg N ha^{-1} d^{-1}) suggests that BNF should be, in principle, capable to fulfill the plant N demand during the entire crop season. However, we found a clear asynchrony between N demand and N supply from BNF from R1 to R5 stages (N gap phase II). Indeed, when the peak of N demand occurred (ca. R5 stage), the contribution from ISN was declining and BNF had not reached its maximum rate yet. A possible carbon shortage to sustain the BNF process may be happening between R1 and R5 where vegetative and reproductive growth overlaps (Walsh et al., 1987). Another speculation can be that low soil temperatures may delay or reduce the rate of BNF (Zhang et al., 1995). Low soil temperatures are expected early in the soybean season in the US North Central regions and especially in early-sown fields as it was the case of the majority of the fields included in this study.

Association between the N gap and the maximum ISN rate (Table 5-3), together with the observation that reduction in BNF is less than proportional in response to increasing ISN (Fig. 5-2), indicates that increases in ISN through soil and management practices (as done in this study through application of N fertilizer) could help mitigate the N limitation in soybean. These results are consistent with Salvagiotti et al. (2009) study, in which different strategies of N fertilization reduced the BNF but still increased total N uptake, leading to an increase in seed yield and seed protein concentration. In contrast, using a large number of cultivars, Santachiara et al. (2017) reported that increasing ISN reduced BNF more than proportionally, resulting in a reduction in accumulated N by R7.

Finally, this study highlighted a number of important topics for future research. First, synchronizing BNF with plant N demand is needed to overcome N limitation in high yield soybean. Second, although high-yield soybean can potentially fix large amounts of N (ca. 300 kg ha^{-1}), this would still not compensate for the seed N removal. As a result, a negative partial N balance (fixed N minus seed N removal) was observed in all zero-N crops in our experiments but one (Smithfield 2016). Third, management practices that increase the ISN would reduce BNF, but tend to increase yield of N limited soybean crops. It would be worth to explore management practices that increase ISN with a relatively small impact upon BNF contribution, although it is uncertain the degree to which these practices would be cost-effective and/or environmentally friendly. Finally, ways to increase BNF by increasing the abundance of the beneficial indigenous rhizobia or increase fixation efficiency are also worth to be explored (Denison, 2012).

Conclusions

There is an asynchrony between BNF and plant N demand. The maximum rate of ISN supply was the most important factor explaining variation in N gap. There was a trade-off between BNF and ISN but the reduction in BNF per unit of additional ISN was less than proportional, indicating that accumulated N and seed yield can be increased through management practices that increase ISN, regardless of BNF. Findings from this study can serve as a base for the development of management practices that reduce N limitation in current and future high-yield soybean production environments.

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CHAPTER 6: FINAL REMARKS AND FUTURE INSIGHTS

The raised question of whether soybean yield is limited by N supply or not was answered in a reductionist way. In chapter two, soybean grown in high-yield environments was limited by N supply. The N limitation increased at a rate of 250 kg per Mg increase in the yield potential from production environments of more than 2.5 Mg ha^{-1}. The N limitation affected seed number and weight, final aboveground dry matter, and seed protein concentration. Full-N crops maintained the same seed protein concentration across the entire yield range (2.5-6.7 Mg ha^{-1}). These initial findings suggested increasing soybean yields due to genetics and agronomic improvements will eventually lead to a larger N limitation. Moreover, this particular work shows that it may be possible to increase seed yield while maintaining seed protein concentration needed for food and feed if N limitation can be overcome. These findings are the initial step to further understanding of more complex scenarios in where other factors, such as water, are limited. It is well known from the literature that water stress can affect N fixation, but the effects of the interaction of water x N stress in soybean is still unknown.

After describing soybean N limitation across environments, the focus turned to high yield environments were N limitation was most likely to occur. Therefore, chapter three evaluated whether seed yield and protein concentration increases due to the N fertilizer applied in full-N were related to increases in N uptake or they were related to effects other than N supply (e.g. luxury consumption, the carbon cost of N fixation process). Also, the variability in N limitation of seed yield was analyzed in the same chapter moving a step forward in the understanding of N limitation in soybean. Seed yield and protein concentration increases in the full N treatment were associated with greater accumulated N without changes in N-use efficiency and/or N harvest index. Indigenous soil N supply modulated the magnitude of the seed yield difference (full-N minus zero-N), indicating that the N limitation is largest in high-yield environments with relatively small indigenous soil N supplies. These findings provided insights into the driver for the N limitation magnitude in high yield soybean and serve as basis to identify environments where N limitations are most likely to occur. While improvements in biological N fixation levels are desired, increases in indigenous soil N supply, due to crop management practices, may also be an alternative to sustain high seed yield and maintain seed protein concentration. Another interesting finding to further explore is the stability of N-use efficiency (in terms of energy) through the N treatments and environments. The biochemical reactions of fixing N from the air demand a lot of energy coming from photosynthesis. However, the stability of the N-use efficiency might indicate that N fixation cost does not affect soybean seed yield and the plant has other mechanisms to support the N fixation cost, such as changes in root:shoot ratio.

In order to improve either biological N fixation or indigenous soil N supply, it is needed to understand the timing of the occurrence N limitation and the underlying physiological mechanisms and processes involved. In chapter four, the higher N accumulation rate, due to fertilizer N supply, supported faster leaf area expansion before R5, greater capture of incident solar radiation, and higher crop growth rate, which, in turn, allowed greater seed setting during the critical period (R3-R6 phase). Similarly, the extra N accumulated before R5 in the full versus zero N treatment resulted in greater availability of N to be remobilized to seed during the seed filling. This allowed the full N treatment to support a similar (or even greater) seed filling rate and seed N concentration compared with the zero N treatment despite the fact that the former has a higher seed number. Hence, any attempt to improve biological N fixation or the amount of indigenous N supply should be focused on the period before the beginning of seed filling where several plant mechanisms are affected. Also, it seems a key point to increase N uptake early in the season to increase seed filling rate and protein concentration.

The dynamics of indigenous soil N supply and biological N fixation were studied in chapter five to further explore why both sources couldn’t meet N demand. It is the first time, indeed, that the dynamics of biological N fixation in high yield environments are contrasted against the crop N demand, and also linked with soybean N limitation. The findings showed an asynchrony between biological N fixation and N demand. Therefore, synchronizing the biological N fixation period with N demand may be desirable but the drivers of this asynchrony are unknown. In that sense, the interaction between indigenous soil N supply and other factors that can affect N fixation, such as soil temperature or photosynthesis, may be worth exploring to elucidate why an early onset of N fixation cannot fix N at high rates. Also, large amounts of soil N at sowing in the whole profile (0-1.2 m) helped to reduce the duration of soybean N limitation regardless of the indigenous soil N supply level. Despite the asynchrony of biological N fixation with N demand, the total amount of N fixed was close to the highest levels reported in the literature. However, high N fixation did not compensate for the amount of N exported in the seeds. From this chapter, we also learned that the trade-off between indigenous soil N supply and biological N fixation is not 1:1. Therefore, increases of indigenous sources can help to mitigate N limitation of high-yield soybean while affecting partially the biological N fixation.

Finally, biological N fixation seems to interact with indigenous N supply and seed yield. This is a triple interaction that still needs to be explored. Relationships between two of these factors (e.g. seed yield and N fixation or soil N and N fixation) can be found in the literature, and often using contrasting environments. However, for a specific environment, for example, the seed yield can be largely modified by improvements in crop management practices, but improvements in indigenous soil N supply, other than N fertilization, will be small. Therefore, a scheme that incorporates the interplay between these three factors (N fixation, seed yield, and indigenous soil N supply) has been developed from this work with the aim of generating future insights in soybean production and the role of soybean in the cropping system (Figure 6-1). All estimations were made based on two assumptions: i) a N requirement of 80 kg of N uptake per metric ton of seed yield produced, and ii) a N harvest index (NHI) of 0.7.

The most common measurement of N fixation in the literature is expressed as a percentage of total N accumulated in the plant at physiological maturity. While this relative measure may lose the insight of absolute magnitude and impact of N fixation, it is an important measurement for calculations of N balance due to: i) the large amount of N exported in the seed as protein, and ii) the non-use of N fertilizer in soybean (except for small amounts applied at sowing as ‘starter’). Theoretically, the percentage of N fixation increases with seed yield following a curvilinear pattern toward a plateau (Figure 6-1 left). This is because of the use of relative values (to the total N uptake) that go from 0 to 100 % and 100% can never be achieved because indigenous soil N supply usually is larger than zero. In absolute terms, this relationship is theoretically linear. Although, we might suspect a decline in absolute values of N fixation at high yield levels if the environments are limited by N supply as some of the experiments in this study. Hence, at the same yield level, a soybean crop grown in a soil with a large contribution of indigenous N supply will fix less N than a soil with a low indigenous N supply contribution (Figure 6-1 left). However, any seed yield improvement due to breeding or crop management will definitely increase the percentage of N fixation in any environment regardless of the level of indigenous soil N supply. This is a desirable crop improvement because of the large amount of N exported in soybean seed and the sustainable goal of maintaining the balance between nutrients exported and inputs to the system (e.g. N balance). Obtaining a near-zero N balance requires matching the percentage of N fixation and NHI, but it may be already possible in environments with low indigenous N supply and current global seed yield level (3-3.5 Mg ha^{-1}; Figure 6-1 right). Also, most progressive farmers with higher yield levels than the average and medium levels of indigenous soil N supply would be reaching a near zero N balance. Improvements in seed yield to > 6 Mg ha^{-1} may allow some of the most productive environments to reach a positive N balance (low and medium indigenous soil N supply), while the same environments but rich in indigenous N supply will not be able to reach a zero N balance unless seed yields approach the biological maximum yield potential of 8 Mg ha^{-1}. Hence, this framework allows predicting the expected improvements in biological N fixation due to seed yield increases in the given situation of indigenous soil N supply of the environment. Finally, it can be also used to predict possible improvement in the N balance of a soybean crop given the seed yield level and the indigenous soil N supply of the production environment.

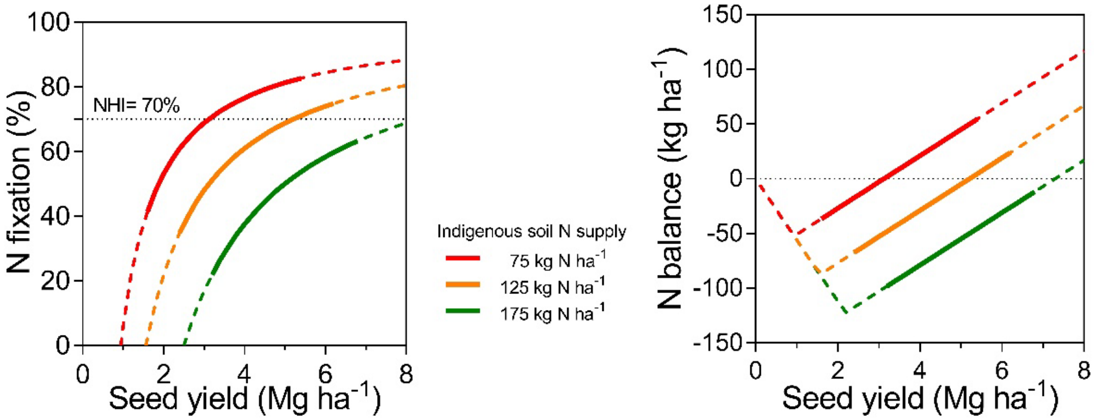

Figure 6-1. Scheme of interplay between soybean seed yield, indigenous soil N supply and N fixation, and their impact upon crop N balance. Dashed lines indicate ranges not well explored in the literature.