

**Study of nitrogen limitation and seed nitrogen sources
for historical and modern genotypes in soybean**

by

Osler Antonio Orteza Amador

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Major Professor
Dr. Ignacio A. Ciampitti

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Abstract

Soybean [*Glycine max* (L.) Merr.] yields have continuously increased over time. Seed yields are determined by the genotype, environment, and management practices ($G \times E \times M$) interaction. Closing yield gaps require a continuous improvement in the use of the available resources, which must be attained via implementation of better management decisions. Linear relationships between seed yield and nitrogen (N) demand are reported in the scientific literature. Main sources of N to the plant are the biological N fixation (BNF) and the soil mineralization processes. On overall, only 50-60% of soybean N demand is met by the BNF process. An unanswered scientific knowledge is still related to the ability of the BNF to satisfy soybean N demand at varying yield levels. Seed N demand not met by N fixation plus soil mineral N, is then fulfilled by the remobilization of N from vegetative organs during the seed filling period. An early remobilization process reduces the photosynthetic activity (leaves) and can limit seed yield. The objectives of this project were to: i) study yield improvements and contribution of N via utilization of contrasting N conditions under historical and modern soybean genotypes, and ii) quantify main seed N sources during the seed filling period. For objective one, four field experiments were conducted during the 2016 and 2017 growing seasons in Kansas, United States (US) and Santa Fe Province, Argentina (ARG). Those experiments investigated twenty-one historical and modern soybean genotypes with release decades from 1980s to 2010s. As for objective two, three field experiments were conducted during the 2015 and 2016 growing seasons in Kansas, US, studying three soybean genotypes: non-roundup ready (RR), released in 1997; RR-1, released in 2009; and RR-2, released in 2014. Across all studies, seeds were inoculated and tested under three N management strategies: i) control without N application (Zero-N); ii) 56 kg N ha⁻¹ applied at reproductive growth stages (Late-N); and iii) 670 kg ha⁻¹

equally split at three timings (Full-N). As for yield improvements and N limitation, soybean yield improvements from the 1980s to 2010s were documented, representing 29% increases in the US and 21% in ARG. Regarding N management, the Full-N fertilization produced a 12% increase in seed yields in the US and 4% in ARG. As for main seed N sources in objective two, remobilization accounted for 59% of seed N demand, and was negatively related to new N uptake occurring during the seed filling period. Seed N demand for greater yields was dependent on both, N remobilization and new N uptake, while for lower yields, seed N demand was mainly supported by the N remobilization process. These results suggest that: a) high seed yields are somehow limited by the availability of N to express their potential, although the question about N application still remains to be fully investigated, as related to the timing and the environment by plant interactions that could promote a N limitation in soybeans; b) remobilization accounts for majority (59%) of N sourced to the seed, and c) high yielding soybean (modern genotypes) rely on diverse N sources: the N remobilization process plus new uptake of N.

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“Mentoring is a brain to pick, an ear to listen, and a push in the right direction.”

~ John C. Crosby

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Dedication

“So do not fear, for I am with you; do not be dismayed, for I am your God. I will strengthen you and help you; I will uphold you with my righteous right hand.” ~ Isaiah 41:10

This completed goal is first of all dedicated to God, who has granted me all the strength and wisdom for being this far. It is also dedicated to my parents, their endless efforts delineated my path here. My wife, for her continuous support, and to my brother for his great friendship and mentorship.

Chapter 1 - General Introduction

Global Production

To meet current projections on food demand, global crop production needs to double by 2050 (FAO, 2009). The projected demand is based on growing population, shift in diets, and increases in the use of biofuels (Pingali, 2006). Projected changes should be accompanied by increases in food production for an ever increased population with ~870 million of chronically undernourished (FAO, 2012). Past investigations have suggested that increasing crop yields per unit area, via intensification, is the most sustainable way to achieve this goal (Green et al., 2005; Godfray et al., 2010; Foley et al., 2011), rather than expanding and adding new lands for production.

Soybean [*Glycine max* (L.) Merr.] is among the top-four major crops, together with maize (*Zea mays* L.), rice (*Oryza sativa*), and wheat (*Triticum aestivum*). Those together account for about two-thirds of the globally harvested crop calories (Tilman et al., 2011). Soybean is the largest source for vegetable oil and animal protein feed in the world (FAO, 2002). Soybean meal in the crushing and oil extraction processes account for approximately 65% of the protein used in feed, and for over 50% of the oilseed, in addition to play an important role in the biodiesel production. In 2017, 118 million hectares were grown and 307 millions of Mg of soybean were produced across the globe (FAO, 2017). Strong soybean needs boost the world's markets, predominantly supplied by the United States, Brazil, and Argentina (Westcott and Jansen, 2016). Soybean production competes for arable land with other crops, although it is not sustainable, expansion is not likely to occur. Hence, increasing yields at existent arable lands has become, as for many other crops, the major driver for addressing the existent demand for soybeans.

Yield Improvements

Yield potential is defined as the maximum attainable yield of a specific crop cultivar at a given environment, with pest and disease well-managed, and without limitation of water and nutrients (Evans, 1993). Ray et al. (2013) studied global improvements of major crops from 1961 to 2008 and found soybean increases of $31 \text{ kg ha}^{-1} \text{ year}^{-1}$, which in turn represented 1.3% annual gain. Similar increases were reported by FAO (2017) and Balboa et al. (2018), exploring a wider spread in time (90+ years). Yield improvement over time has been mainly explained as a result of greater plant biomass and increases in harvest index (Sinclair, 1998; Koester et al., 2014; Gaspar et al., 2017; Balboa et al., 2018) via plant physiological changes such as longer growth in reproductive period (Gay et al., 1980; Zeiher et al., 1982; Kumudini et al., 2001; Egli and Cornelius, 2009; Rowntree et al., 2014; Shen and Liu, 2015), implementation of better management practices (Luedders, 1977; Frederick et al., 1991; Heatherly and Elmore, 2004; Conley and Santini, 2007; Bastidas et al., 2008; Bradley and Sweets, 2008), and genetic advancements (Boerma, 1979; Wilcox et al., 1979; Specht and Williams, 1984; Voldeng et al., 1997; Specht et al., 1999; Foulkes et al., 2009; Rincker et al., 2014; Wilson et al., 2014; de Felipe et al., 2016). Nonetheless, global annual rate for soybean yield increase reported in the last decades (~1.3%) represents only about half of the ~2.4% projected estimations to ensure enough production by 2050 (Ray et al., 2013). Thus, not only for soybeans, a potential production shortage is forecasted if yield gain is not keeping up with the projected demand in the years to come. However, outreach opportunities exist to increase production with a more efficient use of available land and resources (Foley et al., 2011) and to raise yield growth rates through the implementation of better management practices and decisions (Lobell et al., 2009; Mueller et al., 2012).

Nitrogen Demand

Soybean requires large amounts of nutrients, specially nitrogen (N), due to its high protein concentration (~34 to 40% overall) in the seed (Hurburgh et al., 1990; Egli, 1998; Roth et al., 2014; Bellaloui et al., 2015). Among 24 major seed crops, soybean has the greatest N requirement to sustain seed growth, requiring almost three-fold (0.029 g) greater N than corn (0.011 g) or rice (0.010 g) per each gram of photosynthate to produce biomass in seeds (Sinclair and de Wit, 1975). Nevertheless, the almost three-fold difference for N requirement is not as large as the protein difference among the compared crops. In this study, it was observed that soybean crop not only has the greatest N requirement, but also it is in the group of crops producing lower seed biomass per unit of photosynthate, making very challenging to increase seed yields. Due to both, seed composition (high lipid and protein contents) and resource consumption (greatest requirement of N for seed production), soybean is a unique crop and widely differs from other high-yielding groups (i.e. cereal crops) of traditionally low lipids and low protein content. The latter crops, in the other hand, require less use of N to produce photosynthates while also are able to produce more seed biomass per unit of photosynthate (Sinclair and de Wit, 1975).

A recent synthesis-analysis reported increases in N partitioned to the soybean seed, expressed as the N harvest index, with an overall gain over time of 0.0014 per year, 8% of total gain (Balboa et al., 2018), starting as low as 0.66 in the 1930s and raising to 0.72 in the 2010s when yields were slightly above 3000 kg ha⁻¹. Similar partition of N was reported by Bender et al. (2015) with an average of 0.73 at yield levels of 3480 kg ha⁻¹. Looking to even greater yields, 4342 kg ha⁻¹, an increment on the N partition was reported with overall of 0.86 (Mastrodomenico and Purcell, 2012). Increases in seed yield have been associated with lower N concentration in

the seed and consequently lowering the seed protein concentration too (Hartwig and Hinson, 1972; Wehrmann et al., 1987; Rowntree et al., 2013). This effect suggests a limitation of N, especially late in the reproductive stages when the rate of N uptake reaches its peak (Bender et al., 2015; Gaspar et al., 2017). Superior yields are also related to longer duration of leaf area and seed filling period (Gay et al., 1980; Salado-Navarro et al., 1986; Kumudini et al., 2001; Tamagno and Ciampitti, 2017) proposing better environmental conditions and larger N availability. When N supply is smaller than demand, senescence starts, consequently it reduces the photosynthetic activity, and the sum up of these effects limit seed yields (Sinclair and de Wit, 1975). All delineated N background in soybean suggests that maximum yields are dependent on a balanced nutrition, where N is the main limiting factor for not only maximizing yields (Tamagno et al., 2017), but also for maintaining/increasing the seed quality supply (Cafaro et al., 2017).

Nitrogen Sources

The biological N fixation (BNF) process and mineral N in the soil are the main sources for meeting crop N demand in soybean. In overall only 50 to 60% of N demand is met by the BNF process (Salvagiotti et al., 2008; Ciampitti and Salvagiotti, 2018), and in the other hand, it is known that soil N is not able to keep up this large demand (Sinclair and de Wit, 1975). Back then, these authors proposed that any difference between crop N demand and the N offered from BNF + soil N must be obtained from vegetative plant parts. In this sense, in order to meet seed N demand, soybean relies on the plant's ability to store and to remobilize N from the vegetative organs (stems, petioles, leaves, and pod walls) when seeds are growing at a high rate, during the seed filling period (Zeihner et al., 1982; Egli et al., 1983; Kumudini et al., 2002; Sadras and Egli,

2008). Using this framework, N supply to seeds will be influenced by the total N accumulated in the plant before the seed filling and by the new N taken up during the seed filling (Kumudini et al., 2002). As previously described for corn (Ciampitti and Vyn, 2013) and sorghum [*Sorghum bicolor* (L.) Moench] (Ciampitti and Prasad, 2016), the same theoretical framework can be applied to soybeans, with seed N demand as the product of multiple processes interacting together: plant N uptake before the seed filling, N remobilization from vegetative plant parts, new N uptake during the seed filling, N harvest index, and seed yield.

Although a large compendium of scientific and recent literature has reported yield increases over time (Ray et al., 2013; Rowntree et al., 2013; Koester et al., 2014; Gaspar et al., 2017; Balboa et al., 2018), presenting sufficient evidence of yield increases related to greater N demand (Salvagiotti et al., 2008; Gaspar et al., 2017; Tamagno et al., 2017; Balboa et al., 2018), yield responses to N have not been always consistent and often times observed under economical thresholds (Schmitt et al., 2001; Gan et al., 2003; Barker and Sawyer, 2005a; Cafaro et al., 2017; Mourtzinis et al., 2018). A scientific gap is still related to the ability of available N (fixation + soil) to satisfy crop N demand under varying yielding conditions, and implications in the strategies that plants use for sourcing and mobilizing that N to seeds.

Thesis Objectives

Thus, it is valid to hypothesize that yield improvements in the last decades have linearly increased N demand, increasing the likelihood of a N limitation when comparing with relative older soybean materials with lower yield potential. The overall thesis objective was to study yield improvement and contribution of N via utilization of contrasting N conditions under

historical and modern soybean genotypes, and to quantify the main seed N sources during the seed filling period. Specific objectives, per each chapter, were:

Chapter 2:

- Evaluate yield improvement and N limitation under historical and modern soybean genotypes released from the 1980s to the 2010s decades.
- Study the influence of N fertilizer conditions and genotypes released from the 1980s to the 2010s decades on seed yield, seed N removal, and seed protein concentration.

Chapter 3:

- Quantify the contribution of plant organs to the N remobilization process occurring during the seed filling period, R5.5 to R7 reproductive stages, for soybean crop.
- Determine the association between vegetative N, remobilization of N, and N gain during the seed filling, with the utilization of contrasting soybean genotypes and N fertilizer conditions.

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Chapter 2 - Genetic Improvements and Nitrogen Limitation in Historical and Modern Soybean Genotypes

Abstract

The United States (US) and Argentina (ARG) account for over 50% of the global soybean [*Glycine max* (L.) Merr.] production. Soybean nitrogen (N) demand is partially met (50-60%) by the biological N fixation (BNF) process. An unanswered scientific knowledge gap exists on the ability of BNF to fully satisfy soybean N demand at varying yield levels. The overall objective is to explore the potential N limitation at varying N strategies, and for historical and modern soybean genotypes. Four field experiments were conducted during 2016 and 2017 growing seasons in Kansas (US) and Santa Fe (ARG). Twenty-one historical and modern soybean genotypes with release decades ranging from 1980s to 2010s were tested under three N management treatments: i) control without N application (Zero-N); ii) 56 kg N ha⁻¹ applied at R3-R4 growth stages (Late-N); and iii) 670 kg ha⁻¹ equally split at planting, R1, and R3-R4 stages (Full-N). Historical soybean yield gains, from 1980s to 2010s, were 29% in US and 21% in ARG. Following the yield trend, seed N content was superior for modern genotypes in parallel to the reduction on seed protein concentration. Regarding N management, the Full-N produced 12% yield increase in US and 4% in ARG. Yield improvement was primarily related to increases in aboveground biomass, seed number (genotype effect), and in a lesser extent, to individual seed weight (N effect). This study suggests a potential N limitation for soybeans, although there are still questions about the way in which N must be provided to the plant.

Abbreviations: N, nitrogen; BNF, biological nitrogen fixation; MG, maturity group.

Introduction

Soybean [*Glycine max* (L.) Merr.] is considered as the main source for vegetable oil and animal protein feed in the world (FAO, 2002). The United States (US) and Argentina (ARG) account for more than 50% of the global soybean production (USDA-NASS, 2017). In US, more than 85% of the soybean area is in the Corn Belt region, where it is mainly planted in rotation with corn (*Zea mays* L.) (>60%). In ARG, soybeans are primarily planted in the Rolling Pampas and Chaco regions, mainly after wheat (*Triticum aestivum* L.), and after corn in a lesser extent.

Soybean yield potential (Y_p) is genetically determined, and attained under ideal conditions (genotype \times environment \times management practices, $G \times E \times M$), assuming no limitations of water and nutrient supply, and in absence of any biotic (e.g., insects, diseases) and abiotic (e.g., temperature, drought, salinity) yield limiting factors (Evans, 1993). Yield gaps between Y_p and actual on-farm yield (Y_A) are primarily defined by management practices (e.g., planting date, row spacing, nutrient and pest management) and their interaction with the E (soil and weather).

A historical analyses showed that seed yield improved by 246% (1300 versus 3200 kg ha⁻¹) from 1930s to 2010s (Balboa et al., 2018). Annual seed yield increases of 31 kg ha⁻¹ in the US (Specht et al., 1999) and 28 kg ha⁻¹ globally (Wilcox, 2004) were reported from 1970s to 2000s. Rowntree et al. (2013) documented a negative effect on seed protein concentration as yield increased, with a 0.19 g kg⁻¹ decrease per year for maturity group (MG) II and 0.24 g kg⁻¹ decrease for MG III, all relative to the 1920s and 2000s period. Changes in seed yield and seed protein concentration were a consequence of both genetic (Boerma, 1979; Specht and Williams, 1984; Voldeng et al., 1997; Foulkes et al., 2009; Wilson et al., 2014; de Felipe et al., 2016) and

management practices (Frederick et al., 1991; Heatherly and Elmore, 2004; Conley and Santini, 2007; Bastidas et al., 2008; Bradley and Sweets, 2008).

Maximum soybean yields are dependent on a balanced nutrition, with N as the nutrient with largest demand (Sinclair and de Wit, 1975; Egli, 1998; Roth et al., 2014; Bellaloui et al., 2015). Evidence shows that greater seed yields are also associated with larger N requirements (Gaspar et al., 2017; Tamagno et al., 2017; Balboa et al., 2018). The main N sources for the soybean plant are the biological nitrogen fixation (BNF) and soil N mineralization processes. The BNF process is the result of the conversion of atmospheric N₂ into ammonia (NH₃), and later on into N-containing organic components (Wright and Lenssen, 2013). However, only 50 to 60% of soybean N demand is usually met by the BNF (Salvagiotti et al., 2008). Limitation of N for achieving high yields were recently proposed by Wilson et al. (2014), Cafaro et al. (2017), and Ciampitti and Salvagiotti (2018). Following this rationale, research on plant N demand for historical and modern soybean genotypes is a gap in scientific knowledge that needs to be addressed.

It is hypothesized that modern high-yielding soybean genotypes require greater N demand, which might not be solely fulfilled by BNF nor soil N. Thus, N limitation could be limiting the attainable yield or seed protein concentration or both for high-yielding soybean systems. Therefore, the objectives of this study were to: 1) evaluate yield improvement and N limitation under historical and modern soybean genotypes, and 2) study the contribution of contrasting N conditions on primary seed yield components, seed N removal, and seed protein concentration.

Materials and Methods

Experimental Sites Description

Four field experiments were conducted during 2016 and 2017 growing seasons in Rossville, Kansas, United States (US) and Oliveros, Santa Fe, Argentina (ARG). All site-years were planted in corn-soybean rotations. Climate and soil characterization for these sites are presented in Table 2.1. Composite soil samples (10-15 cores) were collected before planting at 15-cm and at 60-cm soil depth in US (39°07'N; 95°55'W). At 15-cm soil depth samples were analyzed for pH (Watson and Brown, 1998); organic matter (OM) (Combs and Nathan, 1998); phosphorus (Mehlich-P) (Beegle and Denning, 1998); cation exchange capacity (CEC); potassium (K), calcium (Ca), and magnesium (Mg) (Warncke and Brown, 1998); and for the soil samples at 60-cm soil depth, only N-nitrate (N-NO₃) concentration was analyzed (Gelderman and Beegle, 1998). In ARG (32°33'S; 60°52'W), all soil samples were collected at 20-cm soil depth, and soil pH, OM, N-NO₃, and Bray P-1 were analyzed using the same methodology conducted for US samples.

Treatments

A combination of 21 genotypes released in different decades and three N fertilizer treatments were evaluated. Soybean genotypes' release decades ranged from 1980 to 2010 and includes MG III and IV in both sites. Within each site, same soybean genotypes were evaluated in both years (Table 2.2). Nitrogen fertilization treatments consisted of three different strategies: i) control with no N applied (Zero-N); ii) late application of 56 kg N ha⁻¹ at the reproductive stages, as described by Fehr and Caviness (1977), beginning of pod, R3, in US and full pod, R4, in ARG (Late-N); and iii) all N provided by fertilizer at a rate of 670 kg N ha⁻¹ equally split at

planting, beginning of flowering (R1), and R3 (US)-R4(ARG) stages (Full-N). Nitrogen treatments were side dressed using liquid Urea Ammonium Nitrate (UAN; N-P-K, 28–0–0), all applied via hand-held backpack sprayer. Prior to planting, seeds were inoculated at commercial rate using liquid Vault® NP (BASF, Ludwigshafen, Germany), active ingredient *Bradyrhizobium japonicum* at 3×10^9 colony forming units per ml, at 62 ml per 23 kg of seed.

All experiments were arranged as a split-plot design with four replications. At the US site, the main-plot was the N treatment and the sub-plot was the genotype factor, while at the ARG site, the main plot was the genotype and N treatment was at the sub-plot factor. The US field plots consisted of four rows spaced at 76 cm with a plot size of 3.0 m wide by 10 m long. The ARG experimental plots had five rows spaced at 52 cm with a plot size of 2.6 m wide by 7.0 m long.

Crop Measurements

Aboveground biomass samples were collected from 1.5 m long in one of the two center rows in all plots before harvest. Abscised and fallen leaves were manually collected during the last sampling, in order to determine total biomass. From each biomass sample, 10 plants were subsampled and fractioned into stems + petioles, leaves, and pod walls. All samples were dried at 65°C until a constant weight was achieved. Total aboveground dry biomass (ADM) was calculated as the sum of the dry weight of plant fractions (stem, leaves, pod walls, and seeds) at beginning of maturity (R7) in ARG, and at full maturity (R8) in the US. Biomass is expressed in kg of dry biomass per ha [Eq. 1].

$$\text{ADM at R7-R8 (kg ha}^{-1}\text{)} = \text{Dry biomass [stem+leaf+pod wall+seed] (kg ha}^{-1}\text{)} \quad (1)$$

At harvest, the two center rows in each plot were harvested with a plot combine. One-kg of seed sample was collected in each plot. Individual seed weight was measured from a 1,000 seed sub-sample. Then, seed number was estimated from the seed weight and seed yield information. Seed yield and seed weight were both adjusted to 0.130 kg H₂O kg⁻¹. Protein concentration (expressed in dry matter basis) was evaluated with the near infrared spectroscopy (NIR) using the samples collected at harvest with a Perten DA 7200 (Perten Instruments, Springfield III, US). From the harvested seed samples, seeds of seven US and four ARG genotypes representing all four release decades (1980s, 1990, 2000s, and 2010s), were ground with a 1 mm mesh and N concentration analysis was conducted (AOAC, 1990). Seed N content, kg ha⁻¹, at harvest was calculated by multiplying the seed dry biomass (kg ha⁻¹) by N concentration (%) following [Eq. 2].

$$\text{Seed N content (kg N ha}^{-1}\text{)} = \text{dry biomass (kg ha}^{-1}\text{)} \times \text{N concentration (\%)} \quad (2)$$

Lastly, pods samples (collected prior to harvest) were dried, mechanically separated into pod walls and seeds, and then weighed for the calculation of the harvest index (HI) parameter, obtained as the ratio of seed biomass related to total aboveground biomass (ADM) at harvest, both expressed in dry basis [Eq. 3].

$$\text{HI} = \frac{\text{Seed biomass (kg ha}^{-1}\text{)}}{\text{Total ADM (kg ha}^{-1}\text{)}} \quad (3)$$

Statistical Analyses

The effect of genotypes, N, and their interaction was tested with a mixed model, fitting the main plant traits evaluated in this study: seed yield, seed number, seed weight, harvest index, dry biomass at R7-R8, seed N content, and seed protein concentration. Genotypes and N were considered as fixed effects, while blocks and years as random effects. Each site (US and ARG) was analyzed independently considering their experimental design. All statistical analyses were performed with the R software (R Software, 2017). As a first step, the Levene's test was conducted using the car package in R program (Fox and Weisberg, 2011) for testing the homogeneity of variance across years for all measured traits. When variances were not homogenous, a model comparison was performed by first, adding the `weight = varIdent` and `correlation = corAR1` functions using the nlme package in R (Pinheiro et al., 2017). Then models were compared using the Akaike information criterion (AIC), Bayesian information criterion (BIC), and the P-value. Analysis of variance (ANOVA) was conducted for each response variable and the results were considered significant when the p-value was smaller than 0.05. Descriptive statistics were conducted on seed yield and the 25th and 75th percentiles (interquartile range), defined two groups: low and high yields. Both groups were then compared with main factors affected by yield changes: seed number, seed weight, and seed protein traits. Following this analysis, regression lines with all observations were plotted for yield vs. mentioned traits. Then, the residuals of those relationships were plotted against the year of release of the respective genotypes to explore real effect of those plant traits over time (adjusted by yield). Regression analyses (Motulsky and Christopoulos, 2003) and figures were executed using Graph Pad Prism 7 Software.

Results and Discussion

Environmental Conditions

Seasonal precipitation, maximum (max), and minimum (min) temperatures were documented throughout both 2016 and 2017 growing seasons at all sites (Table 2.1). Environmental conditions were compared to the 30-year historical mean, and with the last eight-year (2008-2015) seasonal trend. The 2016 and 2017 seasonal mean temperatures were close to the 30-year historical line with approximately only 1°C of deviation (Figure 2.1). As for precipitation, all site-years were within 150 mm range out of the historical line. The 2017 season was the closest to the 30-year historical at both sites. The growing seasons evaluated in this study were comparable to the ones experienced during the last eight-year period (2008-2015).

Yield Improvements and Nitrogen Limitation for Differing Genotypes

Nitrogen fertilizer treatments ($P < 0.05$ in US; and $P < 0.001$ in ARG), genotypes ($P < 0.001$ for both sites), and their interactions ($P < 0.01$ for both) resulted in a significant effect on soybean yields (Table 2.3 and Table 2.4). These results suggested that the magnitude of yield response to N addition differed among genotypes. Yield increases due to the addition of N occurred more frequently with the high-yielding genotypes (modern varieties) and less likely at the low-yielding level (Figure 2.2).

There is large and recent evidence that soybean seed yield has continuously increased over time (Wilcox et al., 1979; Specht and Williams, 1984; Rowntree et al., 2013; Wilson et al., 2014; Balboa et al., 2018). In the US, seed yield ranged from 1998 to 6115 kg ha⁻¹, while ARG ranged from 2210 to 6470 kg ha⁻¹. At both sites, the modern genotypes released in the 2010s decade recorded the highest yield compared to older materials (1980s, 1990s, and 2000s decade)

(Figure 2.2 A-B). When comparing the average yield, across all three N fertilization strategies, for the modern (2010s) versus old (1980s) genotypes, yield increased from 2909 to 4073 kg ha⁻¹ in the US (29% increase), and from 3911 to 4964 kg ha⁻¹ in ARG (21% increase). A similar study contrasting old (low yield potential, released in the 1930s) versus modern (high yield potential, released in the 1990s) genotypes observed a 30% seed yield increase associated with longer duration of green leaf area, and greater dry biomass accumulation for modern genotypes (Kumudini et al., 2001). At a global-scale, seed yield increased from 1100 to 2600 kg ha⁻¹ from 1961 to 2014 (FAO, 2017).

Primary yield drivers resulted from the combination of efforts in plant breeding and fine-tuning on management practices. About half of soybean yield improvement is attributed to the genetic changes, while the other half to improved agronomic practices and their interaction (Rowntree et al., 2013). Among relevant management practices were: earlier planting dates (Conley and Santini, 2007; Bastidas et al., 2008), use of conservation tillage, narrow row spacing, reduction of harvest losses (Heatherly and Elmore, 2004), and improvements in weed management (Bradley and Sweets, 2008). On the other hand, reported genetic improvements in plant traits were disease resistance (Foulkes et al., 2009), elongation (time) of the reproductive stages and, consequently, longer seed filling (Gay et al., 1980), shorter vegetative period favoring longer reproductive periods (Shen and Liu, 2015), and a reduction in lodging (Specht and Williams, 1984). Balboa et al. (2018), summarized yield improvements (from 1922 to 2015) primarily impacting biomass production, resulted from both management practices and genetic improvements.

In general, as related to yield response to the N conditions, when averaged across all genotypes, the greater availability of N (Full-N) presented a positive impact on seed yield at both

sites, US and ARG (Table 2.3 and Table 2.4). The current results follow the rationale that greater seed yields are associated with superior plant N demand, and this (many times) enlarges the gap between crop N uptake and N supplied by the BNF plus soil N. When overall N supply is not enough, the plant will start remobilizing N from vegetative fractions to the seed, causing a reduction in the photosynthetic capacity and thus limiting seed yields (Sinclair and de Wit, 1975). Hence, the addition of inorganic N or fertilizer becomes an alternative for fulfilling N demand and, ultimately, increasing soybean seed yield. In US, the Full-N (670 kg ha⁻¹) increased seed yield by 12% as compared to the Zero-N (without N fertilization, control) (Figure 2.2 C), while Late-N did not statistically differ from the other N treatments. In ARG, the positive response to the Full-N was documented with a 4% increase in seed yields relative to the other N treatments (Figure 2.2 D). For this site, Zero-N and Late-N did not show significant yield differences. In general terms, yield response to the Full-N fertilization was observed among locations, indicating a different potential N limitation to satisfy plant nutrient demand at the explored yield levels (3562 kg ha⁻¹ overall in US and 4330 kg ha⁻¹ overall in ARG) even with yields below the defined threshold for high-yielding soybeans (4500 to 5000 kg ha⁻¹) presented by Salvagiotti et al. (2008). The latter indicates that the yield response to N application is not strictly dependent on the yield environment but other factors affecting the soil N x BNF x plant interaction play a significant role. It has been widely assumed that in high-yielding environments the likelihood of N limitation increased due to the intrinsic positive relationship of N requirement and seed yield (Gaspar et al., 2017; Tamagno et al., 2017). A recent study conducted in Nebraska (US) and Balcarce (ARG) fields with yield levels ranging from 2500 to 6500 kg ha⁻¹ concluded that an overall 11% yield response can occur above 2500 kg ha⁻¹ yielding environments (Cafaro et al., 2017). For the current study, N response was observed only in genotypes yielding above

3240 kg ha⁻¹ in the US and 3702 in ARG (Figure 2.2 C-D) in the control conditions (Zero-N). Similar findings were presented in Wilson et al. (2014), reporting yield response to N fertilizer with modern genotypes (high-yielding) rather than older (low-yielding) counterparts. These results suggest that on these sites, BNF and soil N were not capable to fully meet plant N demand, and thus, yield increases were observed when adding N in a non-limiting approach (Full-N treatment).

As for the Full-N condition, it is worth acknowledging that this strategy was implemented only to address the research question if N was limiting soybean yields and/or protein formation. Although we did not attempt to do any economic and environmental footprint analyses, it is clear that this method will be far-off any profitable and or sustainable threshold. Application of N at early reproductive stages could have a positive impact on seed yield supplementing N to the plant when demand is at a high rate, but some studies have reported no effect from this practice (Gutiérrez-Boem et al., 2004; Barker and Sawyer, 2005). In agreement with these studies, our results showed no significant yield response to Late-N applications (Figure 2.2 C-D) suggesting that a low rate (45 kg ha⁻¹) of N applied during late reproductive stages was not able improve attainable yields in these environments, because of the trade-off between soil N (or fertilizer) and BNF (Salvagiotti et al., 2009).

Biomass, Seed Number, and Individual Seed Weight

Seed yield increase was related to increases in ADM by harvest and in response to both, historical to modern genotypes and to the addition of N as a full rate (Full-N) (Figure 2.3). Previous scientific literature (Kumudini et al., 2001; Balboa et al., 2018) has reported increases on ADM as the main factor driving soybean yield improvements over the last decades. In the

present study, greatest accumulation of ADM was observed with modern genotypes (2010s). On overall, 8600 kg ha⁻¹ of ADM was accumulated by harvest in US, and 9100 kg in ARG (Figure 2.3 A-B). This differential in ADM accumulation in the two locations can be explained by the interaction of factors affecting crop growth such as management practices, differences in genetic traits, weather [precipitation (Muchow, 1985) and temperature (Hadley et al., 1984)], among others. In agreement with the literature findings, ADM accumulation expressed the yield potential of each location, with ARG recording greater ADM (Figure 2.3) and yields (Figure 2.2).

In the current study, the application of the Full-N rate resulted in greater biomass (18% more US and 4% more in ARG) for both locations (Figure 2.3 C-D). A modern study (Bender et al., 2015) observed biomass accumulation increases in response to fertility (N, P, K, S, and Zn) applications, with the biomass gain primarily attributed to an increased rate of dry weight during the late reproductive growth during the seed filling period (from R5 to R7 growth stages).

Moreover, response to genotypes and N was also observed for seed number and individual seed weight. Cafaro et al. (2017) observed seed yield increases associated with superior aboveground biomass, seed number, and seed weight. Biomass improvement for genotypes were mainly related to increases on seed number (upper insets, Figure 2.3 A-B) rather than seed weight (bottom insets, Figure 2.3 A-B), while N additions primarily impacted the individual seed weight (bottom insets, Figure 2.3 C-D) rather than the seed number (upper insets, Figure 2.3 C-D).

Seed number averaged 2532 seeds m⁻² in US and 2563 seeds m⁻² in ARG. Seed number was mainly impacted by genotypes in both sites (upper insets, Figure 2.3 A-B) when comparing contrasting materials' release decades (1980s vs. 2010s). Observations above the 1:1 (y = x) line

showed greater seed number for the 2010s genotypes, with the downside observed with 1980s soybean materials.

In general, greater individual seed weight was observed in ARG with an overall average across all treatments of 169 mg seed⁻¹ relative to 143 mg seed⁻¹ observed in US. This yield component was mainly impacted by the N application at both sites (bottom insets, Figure 2.3 C-D), with Full-N presenting greater seed weight relative to Zero-N conditions. Observations above the 1:1 (y=x) line showed greater individual seed weight for Full-N condition with the downside for the counterpart (Zero-N). At ARG, not only N effect was recorded but also the genotype factor influenced the seed weight. The combined effect of genotype and N showed a significant interaction (Table 2.4), with greatest seed weight observed for the oldest genotype (Williams 82) for the Zero-N treatment (data not shown).

Lastly and in agreement with previous literature (Cafaro et al., 2017), harvest index (HI) was not affected by N treatments. In US site, greater HI was observed with modern genotypes (Table 2.3), same behavior was reported in the synthesis analysis presented by Balboa et al. (2018) observing HI increases over time. In our study, the lowest HI value was 0.30 (P3981, released in 1980) while the maximum was 0.44 (P39T67R, released in 2014).

Nitrogen Exported and Seed Protein Concentration

Historical changes in seed yield presented also implications for changes in N uptake. To achieve high yields, the soybean plant must attain high photosynthesis rates and accumulate large amounts of N in the seeds (Salvagiotti et al., 2008). The N exported in seeds ranged from 115 to 272 kg N ha⁻¹ in US and from 84 to 386 kg N ha⁻¹ in ARG. As was observed for seed yield, modern genotypes (released after 2010) presented the largest amount of N removed (Figure 2.4

A-B). An increase of 19% in US and 23% in ARG were observed comparing overall N removal for modern (2010s) relative to older (1980s) genotypes, averaging all N fertilization conditions.

While N is the main factor determining protein concentration, increases in seed yield are linked to decreases of seed protein concentration due to the dilution effect. The inverse relationship between seed protein concentration and seed yield have been, historically, reported in the scientific literature (Hartwig and Hinson, 1972; Sebern and Lambert, 1984; Wehrmann et al., 1987). Wilcox et al. (1979) observed lower seed protein concentration for modern relative to older genotypes, although, firstly reported for genotypes from MG II released from 1927 to 1974. Moreover, a comprehensive and recent study (Rowntree et al., 2013) explored seed protein concentration changes over 115 different genotypes from MGs II and III released from 1923 to 2008, reporting decreases in seed protein concentration in a linear fashion for both MGs but with a larger reduction (ca. 21%) in seed protein for MG III.

Protein concentration in seed varied between 34 and 45% in US, and between 28 and 44% in ARG. Protein concentration decreased as yield increased over the decades, registering the lowest seed protein concentration levels with the modern, 2000s and 2010s, genotypes (Figure 2.4 C-D). In US, a 3.3% decrease in seed protein concentration in absolute terms was registered, which in turn represented a ~8% in relative terms when comparing 2010s vs. 1980s soybean materials. In ARG, seed protein concentration was reduced by 1.1% in absolute terms and by 3% in relative terms when comparing 2000s and 2010s relative to 1980s and 1990s genotypes. Similar decreases on seed protein concentration (from 41 to 38%) were also observed in a recent study (Cafaro et al., 2017) when seed yield increased from 2500 to 6000 kg ha⁻¹ in response to addition of N fertilizer. The latter results are also consistent with the seed protein

concentration decreases over time for MGs II and III released between 1923 and 2008 (Wilson et al., 2014).

Effect of N applications on the total seed N exported was not observed in US (Table 2.3). Nonetheless, an interaction effect of genotype x N was observed in ARG. The largest amount of seed N exported was a function of the Full-N condition and the modern (NS4955) genotype (data not shown). The Zero-N exported 221 kg ha⁻¹ of seed N, while the Full-N, exported 230 kg ha⁻¹ (Table 2.4), representing a ~4% additional N removal. This additional 4% in seed N content is the N needed for presenting yield response to N addition as suggested by Salvagiotti et al. (2009). The same authors reported an additional 18 kg ha⁻¹ of seed N when comparing control versus the N treatment (264 vs. 282 kg N), suggesting that a ~6% of additional N directed to the seed for increasing yield without inhibiting the BNF process.

As for the seed protein concentration, N treatments effects were not observed at any locations (Table 2.3 and Table 2.4), suggesting that the differential N was not high enough for impacting seed protein concentration. Cafaro et al. (2017), with overall yields of 4500 kg ha⁻¹ found a minor but measurable protein concentration increases (relative ~4%, which in turn represented 1.5% in absolute terms) in soybean in response to the application of N at high rate (330 to 640 kg N ha⁻¹) for both US and ARG. However, the yield levels explored in our study (overall 3946 kg ha⁻¹) were not as high as those reported by Cafaro et al. (2017).

Historical Trends in Relevant Plant Traits: Seed Number, Individual Seed Weight, and Seed Protein Concentration

The analyses of seed yield distribution and separation of the 25th and 75th quartile, regressions lines (yield versus seed number, individual seed weight, and seed protein

concentration), and analysis of residuals of those relationships were performed for each location (Figure 2.5). Low yields for the US site corresponded to observations below 2942 kg ha⁻¹ (25th quartile), while for ARG those yields were below 3468 kg ha⁻¹. On the other side, high yields (75th quartile) in US were above 4100 kg ha⁻¹, while for ARG high yields were above 5060 kg ha⁻¹.

Between locations, similar seed number was observed for the same yielding groups (low and high). Low seed number (~1900 seeds m⁻²) was related to the low-yielding groups, and high seed number (~3250 seeds m⁻²) for the high-yielding group (Figure 2.5 A) in both sites. Strong and positive relationship ($R^2 = 0.81$, $P < 0.0001$, and $n = 214$ in US; and $R^2 = 0.87$, $P < 0.0001$, and $n = 192$ in ARG) was observed between yield and seed number (Figure 2.5 B). In agreement with previous scientific literature (Rotundo et al., 2012), seed number is one of the main yield components increasing with yield. However, different seed yield to seed number slopes were documented for the two sites, showing that ARG attained similar yields with lower seed number (and thus, greater seed weight) relative to US. The residuals of this relationship were plotted against the year of release for all genotypes tested. Lines with deviations from zero are those lines where the P value of the deviation test is smaller than 0.05. Residuals of the seed yield and seed number relationship did not show statistical deviation from zero (Figure 2.5 C) in neither site, suggesting that seed number changes were mainly governed by the productivity-level regardless of the year of release of each genotype.

On overall, higher individual seed weight was observed for ARG (~170 mg seed⁻¹) as compared to US (~140 mg seed⁻¹) (Figure 2.5 D). In US, similar individual seed weight was observed for both low- and high-yielding conditions. In ARG, a slight (~7%) increase in individual seed weight was observed for the high-yielding group. When looking to the yield and

individual seed weight relationship, only ARG showed a positive and significant trend with higher yields related to higher individual seed weight (Figure 2.5 E). In US, the slope of this regression was not different than zero, suggesting no differences in the seed weight among yields groups and supporting the idea that higher yields were primarily related to increases in seed numbers. The residuals of the seed weight and yield did not show any departure from zero for US, but it was slightly different than zero for ARG (Figure 2.5 F); although, slopes were not statistically different between locations. Similar to the seed number result, it was concluded that differences on the individual seed weight were mainly driven by the different yield conditions in both locations rather than an effect of time of release for genotypes. Summarizing, current results are in agreement with results presented by Rotundo et al. (2012), where seed number was clearly the main component driving increases in seed yield in US and ARG.

In general, higher seed protein concentration (~3%, absolute terms), was obtained in US relative to ARG (Figure 2.5 G). The latter result is expected relative to the yield levels attained in each location (yields, ARG>US) and with the already well-documented trade-off between yield and seed protein concentration (Hartwig and Hinson, 1972; Wilcox et al., 1979; Sebern and Lambert, 1984; Wehrmann et al., 1987; Rowntree et al., 2013). At both sites, higher seed protein concentration was attained in the low-yielding groups. However, this effect was magnified for the locations in the US. In the seed yield and seed protein concentration relationships, a significant and negative relation was observed only in US (Figure 2.5 H) suggesting a greater limitation of N, reflected with greater yield increases in US for Full-N vs. Zero-N. Furthermore, this translated into less N allocated to seeds when moving from low- to high-yields. As for the residuals plot of the seed yield and protein concentration relationship, no statistical departures from zero were observed in neither location (Figure 2.5 I). This observation reinforced that yield

and seed protein concentration were predominantly driven by the productivity-level rather than solely year of release for the soybean materials tested.

Results of seed number, individual seed weight, and seed protein concentration suggested: a) differences were important when comparing low- vs. high-yielding groups (Figure 2.5 A-D-G); b) yield differences implied differences (either increases or decreases) on the seed number, seed weight, and seed protein concentration traits where each environment portrayed different strategies for achieving yields (Figure 2.5 B-E-H); and c) changes on seed number, seed weight, and seed protein concentration were mainly governed by the productivity-level rather than by the year of release for the soybean materials (Figure 2.5 C-F-I), yield effect removed by the residual analysis.

Conclusions

Increases in seed yield were documented when comparing the progress from historical (1980s) to modern (2010s) soybean genotypes in the US (+29%) and in ARG (+21%). Historical changes in seed yield were also reflected in the seed N removal, that increased by +19% in US and by 23% in ARG. Seed protein concentration was decreased as productivity increased, 3.3 and 1.1% decreases (in absolute terms) for US and ARG, respectively, comparing modern to historical genotypes.

Seed yield response to N application, Full-N (without limitation of N) vs Zero-N (control), varied between 12% in US to 4% in ARG. In US, additional 17 kg N ha⁻¹ of seed N content was required to increase yields, while in ARG, this seed N content was of 9 kg N ha⁻¹. The application of late-season N, 56 kg N ha⁻¹ applied at R3-R4, was not overcoming the

potential N limitation documented when comparing seed yield of Full-N versus seed yield of the Zero-N condition.

From a historical perspective, the genotype effect was reflected as yield improvement over time, primarily obtained from changes in biomass and seed number. From a N nutritional standpoint, N fertilization primarily impacted seed weight, pinpointing that main N limitations might be encountered during the seed filling period if conditions are favorable for maximum yield.

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Figures

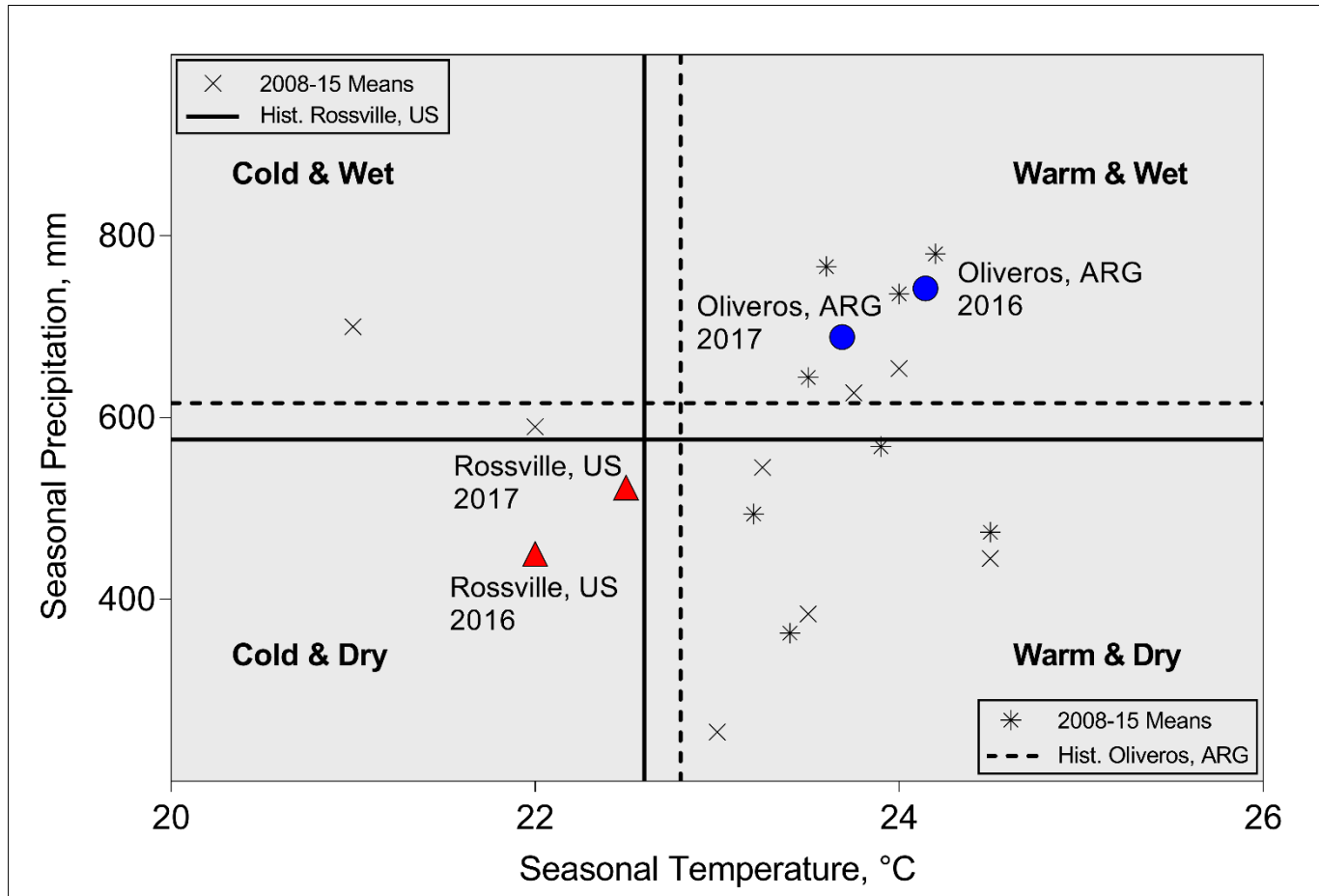


Figure 2.1 Thirty-year historical mean, 2008 to 2015 seasonal trends, and 2016-2017 growing season means for temperature and precipitation characterization at Rossville, US and Oliveros, ARG. Red triangles: US 2016 and 2017; Blue circles: ARG 2016 and 2017; (×) 2008 to 2015 US trend; (*) 2008 to 2015 ARG trend; solid lines: 30-year historical mean for US; and dashed lines: 30-year historical mean for ARG.

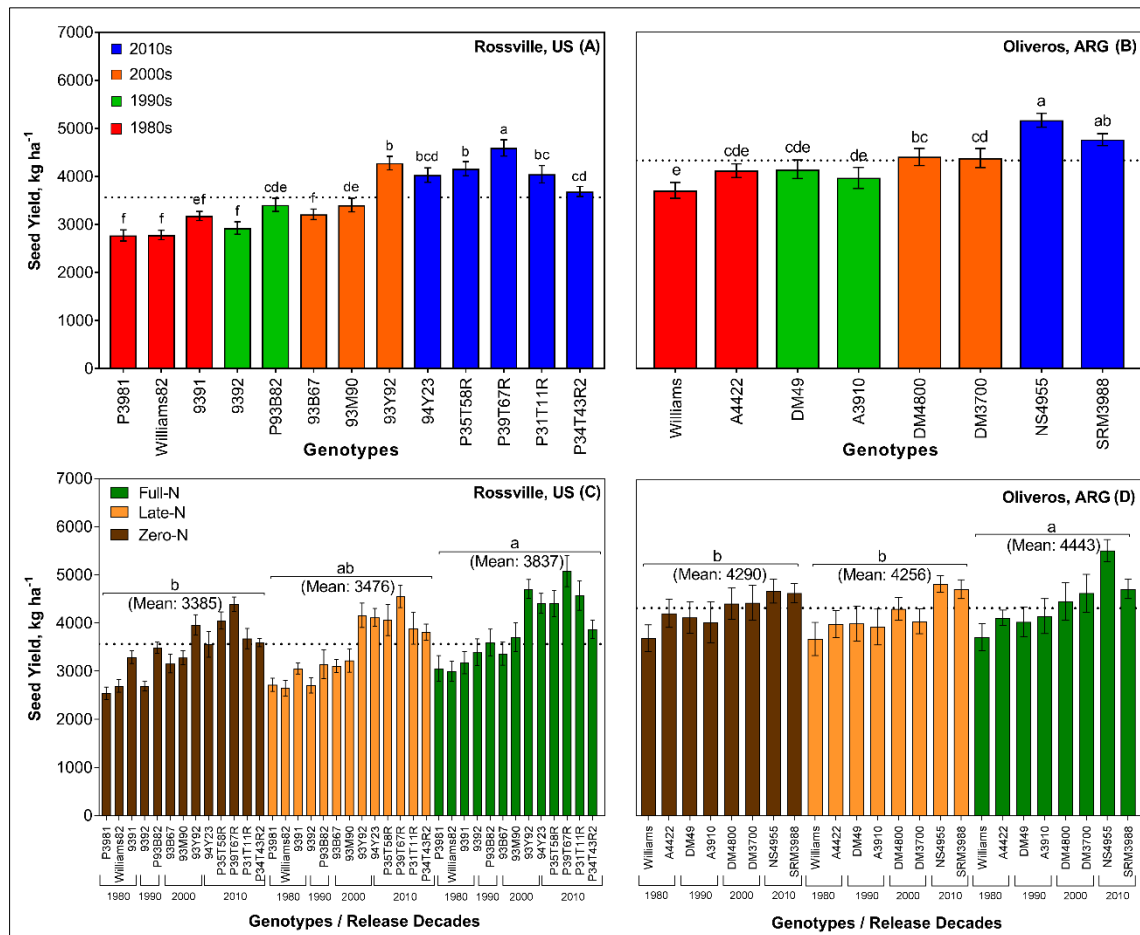


Figure 2.2 Seed yield (kg ha^{-1}) for 21 historical and modern soybean genotypes as general mean of three N fertilization rates (A-B), and seed yield for three N rates and 21 genotypes (C-D) during the 2016 and 2017 growing seasons at Rossville, US and Oliveros, ARG. Upper panel: colors represent genotypes of four released decades: 1980s (red); 1990s (green); 2000s (orange); and 2010s (blue). Bottom panel: colors represents three nitrogen rates: Zero-N (brown); Late-N (yellow); and Full-N (green). Each bar shows the mean and standard errors of the mean. Dashed lines show the overall mean for each site. Different letters indicate significant differences at $P \leq 0.05$.

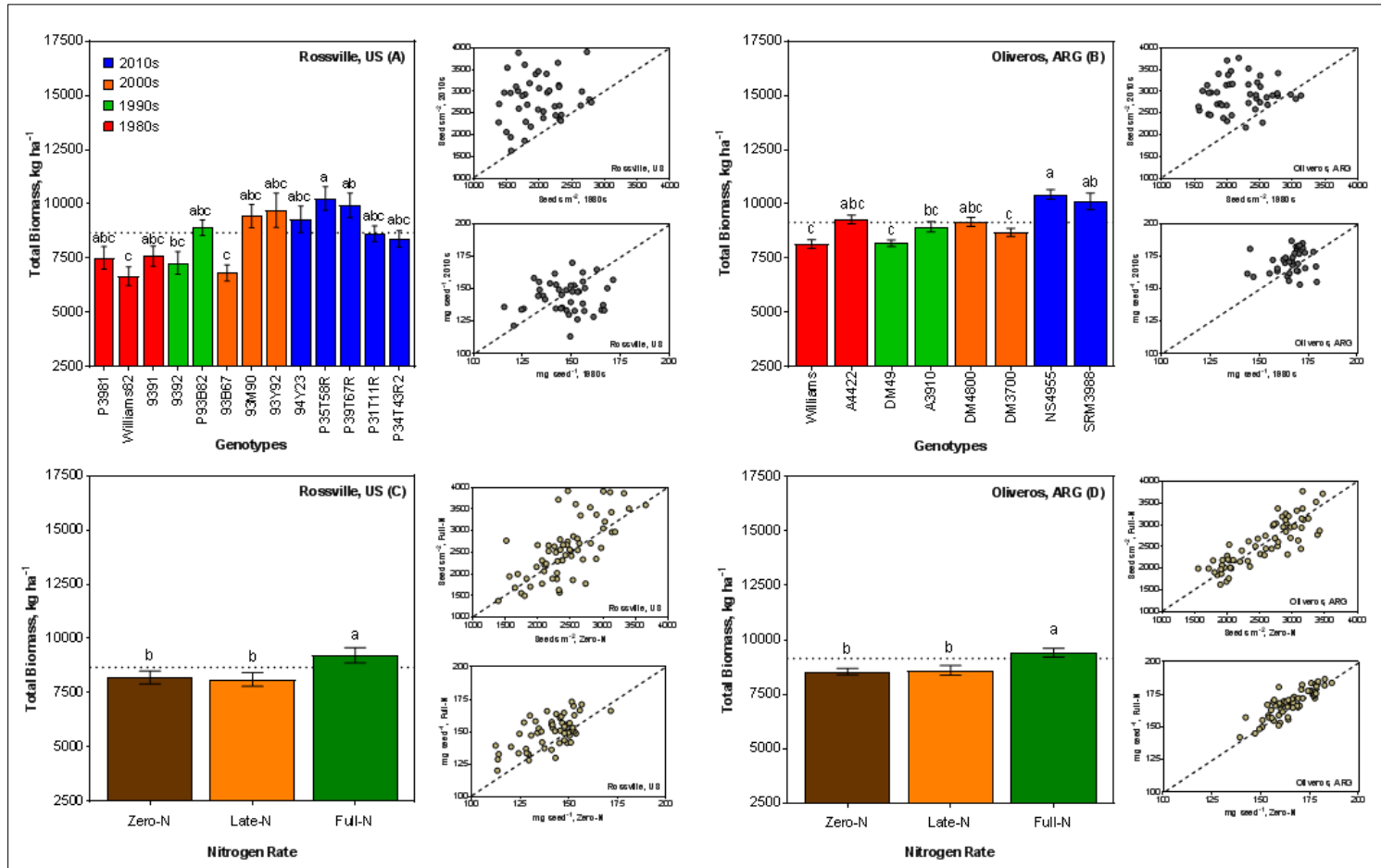


Figure 2.3 Total aboveground biomass (kg ha⁻¹) for 21 historical and modern soybean genotypes (A-B), and three N rates (C-D) at Rossville, US and Oliveros, ARG during the 2016 and 2017 growing seasons. Upper panel: colors represent genotypes of four released decades: 1980s (red); 1990s (green); 2000s (orange); and 2010s (blue). Bottom panel: colors represents three nitrogen rates: Zero-N (brown); Late-N (yellow); and Full-N (green). Each bar shows the mean and associated standard errors of the mean. The dashed lines show the overall mean at each site. Different letters indicate significant differences at $P \leq 0.05$. Scatter plot insets on (A-B) compare seed number (seeds m⁻²) and individual seed weight (mg seed⁻¹) for contrasting genotypes (1980s versus 2010s), and insets on (C-D) compare the same traits for contrasting N rates (Zero-N versus Full-N). Dashed lines in all scatter plot insets show the 1:1 ($y = x$) lines.

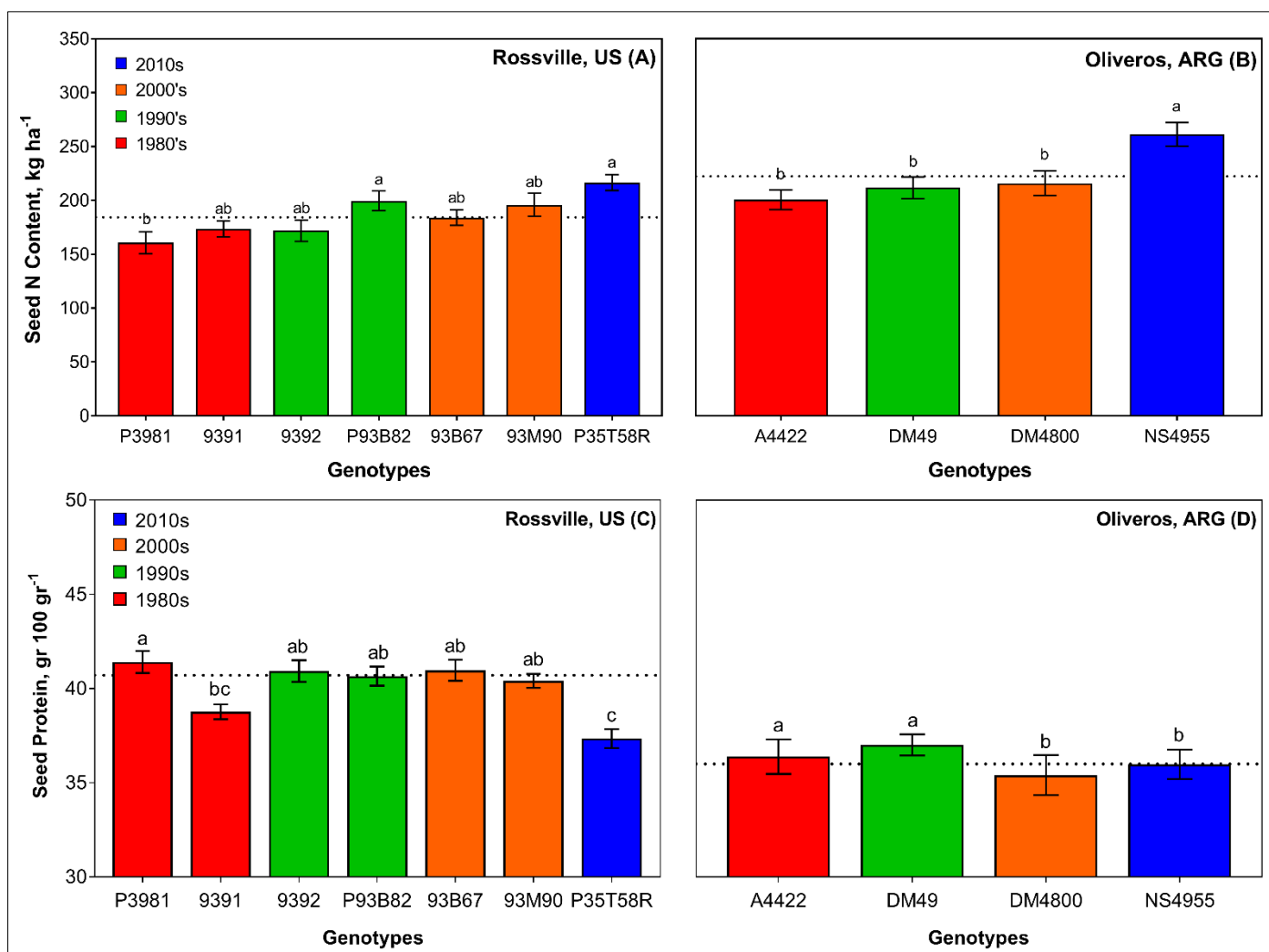


Figure 2.4 Seed nitrogen content (kg ha⁻¹) and seed protein concentration (gr 100 gr⁻¹ seed), both in dry basis, for 21 historical and modern soybean genotypes of different release decades as general mean of three N fertilization rates for Rossville, US and Oliveros, ARG during the 2016 and 2017 growing seasons. Each color groups genotypes in four release decades: 1980s (red); 1990s (green); 2000s (orange); and 2010s (blue). Each bar shows the mean and associated standard errors of the mean. The dashed lines show the overall mean for each location. Different letters indicate significant differences at $P \leq 0.05$.

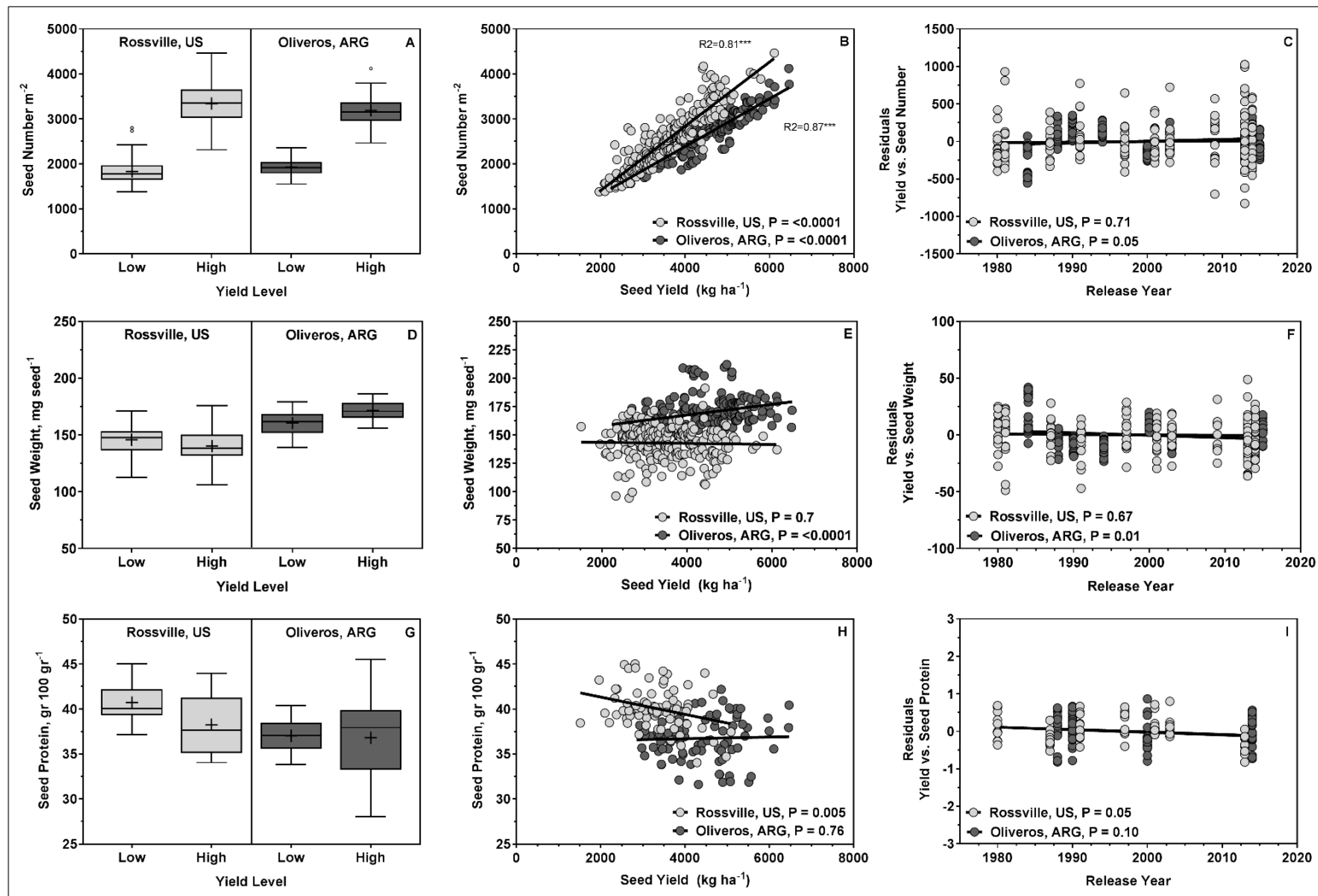


Figure 2.5 Seed number (seeds m⁻²; A, B, C), seed weight (mg seed⁻¹; D, E, F), and seed protein concentration (gr 100 gr⁻¹; G, H, I) for Rossville, US and Oliveros, ARG during the 2016 and 2017 growing seasons. First column (left): first (25th, low yields) and third (75th, high yields) percentiles comparisons; second column (center): seed yield (kg ha⁻¹) and trait regressions; and third column (right): residuals of the seed yield and trait relationships plotted against year of release for genotypes.

Tables

Table 2.1 Soil and climate characterization for 2016 and 2017 growing seasons in Rossville, United States (US) and Oliveros, Argentina (ARG).

Year	Location†	Coordinates	Precipitation (mm)	T Max (°C)	T Min (°C)	Soil pH	CEC Meq 100 g ⁻¹	OM (%)	N-NO ₃ ppm	Mehlich-P ppm‡	K ppm	Ca ppm	Mg ppm
2016	Rossville, US	39° 07' N; 95° 55' W	450	28	16	6.9	11.0	2.2	3.0	21	153	2074	202
	Oliveros, ARG	32° 33' S; 60° 52' W	742	31	17	5.5	-	2.1	6.3	12	-	-	-
2017	Rossville, US	39° 07' N; 95° 55' W	523	29	16	7.3	5.8	1.3	2.7	13	90	951	95
	Oliveros, ARG	32° 33' S; 60° 52' W	688	28	19	-	-	2.52	23.5	9.5	-	-	-

† At ARG, all soil samples were collected at 20 cm of depth; 2015-2016 and 2016-2017 growing seasons for this location.

‡ P test conducted was Bray P-1 instead of Mehlich-P.

Table 2.2 Description of field experiments conducted in Rossville, Kansas, United States (US) and Santa Fe Province, Argentina (ARG) during the 2016 and 2017 growing seasons.

Year	Location†	Genotype‡	Release Year	Maturity Group	Planting Date
2016	Rossville, US	P3981, Williams82, 9391, 9392, P93B82, 93B67, 93M90, 93Y92, 94Y23, P35T58R, P39T67R, P31T11R, and P34T43R2	1980, 1981, 1987, 1991, 1997, 2001, 2003, 2009, 2013, 2013, 2013, 2014, and 2014	3, 3, 4, 3.8, 3.8, 3.9, 3.9, 3.9, 4, 3, 4, 3.1, and 3.4	May, 12th
	Oliveros, ARG	Williams, A4422, DM49, A3910, DM4800, DM3700, NS4955, and SRM3988	1984, 1988, 1990, 1994, 2000, 2003, 2014, and 2015	3, 4, 4, 3, 4, 3, 4, and 3	Nov, 9th
2017	Rossville, US	P3981, Williams82, 9391, 9392, P93B82, 93B67, 93M90, 93Y92, 94Y23, P35T58R, P39T67R, P31T11R, and P34T43R2	1980, 1981, 1987, 1991, 1997, 2001, 2003, 2009, 2013, 2013, 2013, 2014, and 2014	3, 3, 4, 3.8, 3.8, 3.9, 3.9, 3.9, 4, 3, 4, 3.1, and 3.4	May, 18th
	Oliveros, ARG	Williams, A4422, DM49, A3910, DM4800, DM3700, NS4955, and SRM3988	1984, 1988, 1990, 1994, 2000, 2003, 2014, and 2015	3, 4, 4, 3, 4, 3, 4, and 3	Nov, 14th

† 2015-2016 and 2016-2017 growing seasons for ARG location.

‡ Each position for genotype is related to its respective position on released year and maturity group.

Table 2.3 Overall means and analysis of variance (ANOVA) for nitrogen (N) and genotypes on seed yield, seed number, seed weight, harvest index (HI), aboveground dry biomass (ADM) at R8, N exported in seed, and seed protein at harvest for Rossville (US) field experiments as summary of the 2016 and 2017 growing seasons.

Genotype, Release Year	N Rate	Seed Yield	Seed Number†	Seed Weight	Harvest Index	ADM R8‡	Seed N Content	Seed Protein
		kg ha ⁻¹	Seeds m ⁻²	mg seed ⁻¹	%	kg ha ⁻¹	kg ha ⁻¹	g 100 g ⁻¹
P34T43R2, 2014		3685	2537	145	0.41	8255	.	.
P31T11R, 2014		4019	2901	140	0.44	8509	.	.
P39T67R, 2013		4750	3366	137	0.42	9935	.	.
P35T58R, 2013		4216	2768	143	0.38	10250	205	38.6
94Y23, 2013		3894	2872	142	0.38	9638	.	.
93Y92, 2009		4204	3073	140	0.37	10092	.	.
93M90, 2003		3486	2354	143	0.36	9185	196	40.3
93B67, 2001		3016	2260	143	0.42	7397	184	40.5
P93B82, 1997		3598	2384	146	0.35	8904	200	40.5
9392, 1991		2882	2134	137	0.36	7926	172	40.0
9391, 1987		3152	2249	143	0.37	8395	174	39.1
Williams82, 1981		2702	1967	144	0.37	7325	.	.
P3981, 1980		2753	1926	145	0.30	8533	161	41.0
	i) Zero-N	3385	2465	140	0.38	8176	176	39.9
	ii) Late-N	3476	2469	141	0.39	8237	.	.
	iii) Full-N	3837	2634	147	0.37	9975	193	40.1
N Rate		<0.05*	ns	<0.01**	ns	<0.01**	ns	ns
Genotype		<0.001***	<0.001***	ns	<0.05*	<0.001***	<0.001***	<0.001***
N Rate × Genotype		<0.01**	ns	ns	ns	ns	ns	ns

† ns, nonsignificant at the 0.05 probability level.

‡ Fallen leaves were collected from the ground for biomass estimation at the R8 stage.

Table 2.4 Overall means and analysis of variance (ANOVA) for nitrogen (N) and genotypes on seed yield, seed number, seed weight, harvest index (HI), aboveground dry biomass (ADM) at R7, N exported in seed, and seed protein at harvest for Oliveros (ARG) field experiments as summary of the 2016 and 2017 growing seasons.

Genotype, Release Year	N Rate	Seed Yield kg ha ⁻¹	Seed Number† seeds m ⁻²	Seed Weight mg seed ⁻¹	Harvest Index %	ADM R7‡ kg ha ⁻¹	Seed N Content kg ha ⁻¹	Seed Protein g 100 g ⁻¹
SRM3988, 2015		4816	2745	175	0.40	10334	.	.
NS4955, 2014		5200	3149	164	0.42	10445	259	35.3
DM3700, 2003		4353	2589	168	0.44	8694	.	.
DM4800, 2000		4389	2446	179	0.41	9169	210	35.1
A3910, 1994		3912	2484	157	0.38	8947	.	.
DM49, 1990		4120	2596	158	0.44	8187	206	37.4
A4422, 1988		4144	2507	165	0.38	9281	212	37.3
Williams, 1984		3705	1983	188	0.39	8152	.	.
	i) Zero-N	4290	2568	168	0.41	9051	221	36.4
	ii) Late-N	4256	2512	169	0.41	8983	215	36.2
	iii) Full-N	4443	2607	170	0.41	9420	230	36.3
N Rate		<0.001***	<0.01**	<0.001***	ns	<0.01**	<0.01**	ns
Genotype		<0.001***	<0.001***	<0.001***	ns	<0.001***	<0.001***	<0.001***
N Rate × Genotype		<0.01**	ns	<0.01**	ns	ns	<0.001***	ns

† ns, nonsignificant at the 0.05 probability level.

‡ Only leaves present in main stem at the R7 stage were included.

Chapter 3 - Nitrogen Sources and Demand During the Soybean Seed Filling Period: Genotype and N fertilization

Abstract

Soybean [*Glycine max* (L.) Merr.] seed nitrogen (N) demand not met by N fixation + soil, is fulfilled by N gain from new uptake and N remobilization from vegetative organs during the seed filling period. The objectives of this study were: i) quantify the contribution of plant organs to N remobilization from R5.5 to R7 growth stages, during seed filling, and ii) determine the association between N remobilization and N gain during this timeframe. Three field experiments were conducted during 2015 and 2016 growing seasons in Kansas, US. Three soybean genotypes were utilized: non-RR, released in 1997; RR-1, released in 2009; and RR-2, released in 2014. All tested under three N management: i) control without N application (Zero-N); ii) 56 kg N ha⁻¹ applied at the R3 growth stage (Late-N); and iii) 670 kg ha⁻¹ (Full-N, equally split at planting, R1, and R3 stages). Aboveground biomass samples were collected at the R5.5 and R8 stages, plants were fractioned in stems + petioles, leaves, pod walls, and seeds. The modern genotype out yielded (267 kg ha⁻¹, 9%) older materials. Nitrogen fertilized treatments increased yields by 9% (256 kg ha⁻¹) relative to the Zero-N. Treatment differences were neither observed for N remobilization nor for N gain. Remobilization accounted for 59% of seed N demand, driven by plant size at R5.5, and was negatively related to N gain during seed filling. Nitrogen demand for greater yields was dependent on both, N remobilization and N gain, while lower yields were mainly supported by the N remobilization process.

Abbreviations: N, nitrogen; BNF, biological nitrogen fixation; SFP, seed filling period.

Introduction

Soybean [*Glycine max* (L.) Merr.] is the largest and more important oilseed crop in the world due to its relevance as oil and protein source for human consumption and animal feed. Soybean yield have significantly increased through the history, primarily due to the combination of genetic improvement, crop management, and rapid technology adoption (Specht et al., 1999). A historical review with data compiled from published literature documented a seed yield improvement of 246% (1.3 versus 3.2 Mg ha⁻¹) from the 1930s to the 2010s, with improvements largely attributed to increases in plant biomass (Balboa et al., 2018).

In soybean, seed yield is linearly related with total N uptake (Salvagiotti et al., 2008; Tamagno et al., 2017; Ciampitti and Salvagiotti 2018). The plant N demand is largely allocated to the seed at harvest (71%) and its partially fulfilled by two main sources: i) biological nitrogen fixation (BNF) and ii) soil mineral N – as exogenous N sources. Total seed N demand is the result of the interaction between plant N uptake before the seed filling period (SFP), N remobilization from vegetative organs, N acquired during the SFP, seed yield and the partitioning of N to the seeds (i.e., N harvest index; NHI). The same theoretical framework has been described for other crops such as sorghum (Ciampitti and Prasad, 2016) and corn (Ciampitti and Vyn, 2013).

Most of the total N in seeds at harvest is largely provided by the N remobilized from vegetative plant fractions (Egli et al., 1978 [20-60%]; Hammond et al., 1951 [58-64%]; Kumudini et al., 2002 [75-92%]; Zeiher et al., 1982a [30-100%]). Nitrogen remobilization can be quantified as the difference between the total amount of N in non-seed tissues from the beginning to the end of the seed filling period, R5 to R7 (Fehr and Caviness, 1977). However, similar remobilization analyses were performed calculating N remobilization from as early as R4

(Kumudini et al., 2002) or as late as R5.5 (Poeta et al., 2014). Sinclair and de Wit (1976) postulated that N remobilization from vegetative organs to the seeds occurs when seeds are growing at a high rate, resulting in a high N demand that cannot be satisfied solely by N uptake (i.e., BNF + soil N) during the SFP. Using this framework, N supply to the seeds will be affected by i) total N accumulated prior to seed filling (R5), and ii) N taken up by the crop from the R5 until the R8 reproductive stages (Kumudini et al., 2002).

A review analysis (Salvagiotti et al., 2008) in soybean concluded that the difference between N uptake (from soil) and N offered by the BNF process tends to increase at high yielding levels, since greater yields are strictly associated with greater N demand. Thus, high-yielding modern soybean genotypes require a greater N demand (Balboa et al., 2018) that might not be fully provided by both BNF and soil mineral N. The interaction between soybean genotypes differing in yield potential and management N strategies for fulfilling N demand, have not yet been reported.

Hence, the hypothesis of this study investigates the addition of exogenous N via fertilization for addressing N limitation in soybean, primarily during the seed-filling period. Therefore, the objectives were to: i) quantify the contribution of plant organs to N remobilization during the seed-filling period, R5.5 to R7 (including leaves) and ii) determine the association between N remobilization and N gain during this timeframe, with the utilization of historical soybean genotypes (released years: 1997, 2003, and 2014) and fertilization N strategies (Zero-N, Late-N addition, and non-limited by N).

Materials and Methods

Experimental Sites description

Field experiments were conducted at Ashland Bottoms Research Center (39° 8' N, 96° 37' W) and at Ottawa East Central Experimental Field (38° 32' N, 95° 14' W) during the 2015 season, and during the 2016 season at Ottawa (East Central Experimental Field), all sites located in Kansas, US. Soil and climate characterization for all sites are presented in Table 3.1.

Composite soil samples were collected before planting in each location at 60 cm soil depth for N-NO₃ analysis (Gelderman and Beegle, 1998) and at 15 cm soil depth for pH (Watson and Brown, 1998), Mehlich P (Beegle and Denning, 1998), organic matter (OM) (Combs and Nathan, 1998), cation exchange capacity (CEC), K, Ca, and Mg (Warncke and Brown, 1998).

Soil type at Ashland was moderately well drained, with reduced slopes (0-11%), while at Ottawa was characterized as a deep soil, poorly drained and with slopes ranging from 0 to 3%.

Treatments

A combination of three genotypes and three N fertilization strategies were evaluated at all three environments. Historical genotypes evaluated were: i) P93B82 (genotype 1), a maturity group (MG) 3.8 and non-RR material released in 1997; ii) 93Y92 (genotype 2), a MG 3.9 and a RR-1 material released in 2009; and iii) P34T43R2 (genotype 3), a MG 3.4 and a RR-2 material released in 2014, all DuPont Pioneer (Johnston, IA) soybean genotypes. Fertilization with N treatments consisted in three different strategies: i) control with no N applied (Zero-N); ii) late application of 56 kg N ha⁻¹ (Late-N); and iii) all N provided by fertilizer at a rate of 670 kg N ha⁻¹ (Full-N; equally split at planting, R1 and R3 stages). Nitrogen treatments were side dressed in the soil surface with liquid urea–NH₄NO₃ (UAN; N-P-K 28–0–0) and all applied via hand-held

backpack sprayer. Seeds were inoculated at commercial rate (liquid Vault[®] NP, 62 ml per 23 kg of seed) prior to planting. All experiments were arranged as a completely randomized block design with three replicates in each site. Field plots were four rows spaced at 76 cm with 3.05 m wide by 15.2 m long at all sites.

Crop Measurements

Aboveground biomass samples were collected from 1.5 m long in one of the two center rows in all plots at the reproductive stage R5.5, beginning of the linear seed filling phase with approximately 84% of seed moisture content (Poeta et al., 2014), and before harvest maturity, reproductive stage R8. From each collected sample, 10 plants were subsampled and fractioned into stems + petioles (herein term as stem), leaves (including fallen leaves in the sampling area in R8), and pods at both sampling stages. For the biomass sampling at the R8 stage, fallen leaves were collected in order to have a biomass estimation and thus able to determine N remobilization, herein this sampling stage will be termed as R8*. Pods were dried and mechanically separated into pod walls and seeds at both sampling times using a stationary thresher machine. All samples were dried at 65°C until constant weight and ground with a 1 mm mesh for N concentration analyses conducted via combustion method for N (AOAC, 1990).

At harvest, 28 square meters in each plot were harvested with a combine. Seed weight was measured from 1 kg of seed sample and seed number was estimated from the seed weight and yield. Seed yield and seed weight were both adjusted at 0.130 kg H₂O kg⁻¹ seed moisture content.

Data calculations and parameters evaluated

Nitrogen content in each plant fraction (stem, leaf, pod wall, and seed) and in each stage was calculated as the product of dry biomass (BM) multiplied by its N concentration (%N) [Eq. 1]. Total vegetative N content included the N in stem, leaves, and pod walls.

$$\text{N Content (kg ha}^{-1}\text{)} = \text{BM}_{\text{fraction}} \text{(kg ha}^{-1}\text{)} \times \%N_{\text{fraction}} \quad (1)$$

Apparent N remobilization, herein term as N remobilization, was calculated as the difference between N (kg ha⁻¹) in each vegetative fraction (stem, leaf, and pod wall) at R5.5 and N present in the same structures at R8* [Eq. 2] (Egli et al., 1983; Egli and Leggett, 1973; Kumudini et al., 2002; Zeiher et al., 1982). For instance, N remobilization from the stem is the product of total N content in stem at R5.5 minus total N content in stem at R8*. Same rationale was followed with leaf and pod wall. Total N remobilization was calculated as the sum of all remobilized N in the three vegetative plant fractions (stem, leaf, and pod wall).

$$\text{Remobilized N (kg ha}^{-1}\text{)} = [\text{Vegetative N at R5.5} - \text{Vegetative N at R8*}] \text{(kg ha}^{-1}\text{)} \quad (2)$$

The remobilization contribution of N from each vegetative fraction (i.e. stem, leaf, or pod wall) was calculated as relative to the total remobilized N [Eq. 3].

$$\text{Remobilization contribution per fraction (\%)} = \frac{\text{Remobilized N in fraction (kg ha}^{-1}\text{)}}{\text{Total N remobilized (kg ha}^{-1}\text{)}} \times 100 \quad (3)$$

Proportion of seed N at maturity provided by the N remobilization process is the result of dividing the total apparent remobilized N to the total N in the seed at maturity, R8* stage [Eq. 4].

$$\text{Proportion of remobilized N in the seed (\%)} = \frac{\text{Total remobilized N (kg ha}^{-1}\text{)}}{\text{Total N in seed at R8* (kg ha}^{-1}\text{)}} \times 100 \quad (4)$$

Nitrogen gain was determined as the total N content achieved at R8* in the plant minus its N content at R5.5 [Eq. 5].

$$\text{Nitrogen gain (kg ha}^{-1}\text{)} = \text{Total N at R8* (kg ha}^{-1}\text{)} - \text{Total N at R5.5 (kg ha}^{-1}\text{)} \quad (5)$$

Harvest index (HI) was determined dividing seed biomass to total aboveground biomass (BM) at R8* [Eq. 6].

$$\text{HI} = \frac{\text{Seed biomass (kg ha}^{-1}\text{)}}{\text{Total BM (kg ha}^{-1}\text{)}} \quad (6)$$

Statistical Analyses

For all statistical analyses, genotype and N fertilization were considered as the fixed effects with blocks and site-years treated as random effects. All statistical analyses were performed with the R software (R Software, 2017). The Levene's test was conducted using the car package in R program (Fox and Weisberg, 2011) for testing homogeneity of variance within and across sites for yield and all measured variables. When variances were not homogenous, a model comparison was performed by first, adding the weight = varIdent and correlation =

corAR1 functions using the nlme package in R (Pinheiro et al., 2017). Then models were compared using the Akaike information criterion (AIC), Bayesian information criterion (BIC), and the p-value. Analysis of variance (ANOVA) was conducted for each factor and treatment means were considered significant when the p-value of the evaluated factor was <0.05. Figures were plotted using Graph Pad Prism version 7.0 for Windows (GraphPad, 2017). Lastly, descriptive statistics analyses were conducted on yield and seed N sources to explore dependencies on N processes (i.e. remobilized N versus N gain proportion) during the seed filling for contrasting genotypes and yield levels and to explore main N reservoirs in the plant comparing low and high N content conditions at the R5.5 stage.

Results and Discussion

Nitrogen Fertilization and Genotype Effects on Total Biomass and Seed Yield

Across site-years, overall seed yield was of 2750 kg ha⁻¹, with genotype 3 yielding 9% more (267 kg ha⁻¹) relative to rest of the genotypes (Table 3.2). Likewise, greater yields for modern genotypes have been documented in the literature (Specht et al., 1999; Kumudini et al., 2002; Balboa et al., 2018) relative to older soybean materials. For fertilization, Full-N and Late-N presented a 9% increase in seed yield (256 kg ha⁻¹) relative to the control. Studies in the scientific literature proposed that soybean rarely respond to N fertilization unless high N rates are supplied or nodulation activity is affected (Salvagiotti et al., 2008). However, soybean yield responses to very high levels of N fertilization that overcome the trade-off between soil N and BNF were documented in previous studies (Thies et al., 1995; Gan et al., 2003; Mourtzinis et al., 2015; Cafaro et al., 2017). In the present study, soybean yields were equally impacted by the

Full-N and Late-N treatments (Table 3.2), suggesting the need of a small N amount to increase seed yield, primarily with the nutrient provided later in the growing season.

Biomass accumulation did not show any statistical differences among treatments at the R5.5 stage ($P>0.05$) but genotypes 2 and 3 presented greater biomass at the R8* stage for seed and total biomass plant fractions relative to the genotype 1. Both N applications (Late-N and Full-N) increased all plant biomass fractions (stem, leaf, pod wall, and seed) at the R8* stage (Table 3.2).

As for the yield components, genotype 3 presented greater seed number, 2103 seeds m^{-2} in absolute terms, which represented 13% more, relative to other genotypes (Table 3.2). As for N conditions, the Late-N treatment resulted in greater seed number (2019 seeds m^{-2}), 11% more, relative to the Zero-N treatment. The Full-N treatment did not differ from the Late- and Zero-N conditions resulting in 1941 seeds m^{-2} . High yields are primarily accompanied by improvements in seed number rather than seed weight. Differences in seed weight were only attributed to genotypic effects, with greater values for genotypes 1 and 2 (151 and 140 mg seed $^{-1}$). Harvest index, averaged 0.32 units, without presenting any statistical differences among all treatments.

Nitrogen Content and Concentration in Plant Fractions

No interaction was observed for N content at both stages. At R5.5 stage, soybean accumulated 201 kg N ha^{-1} without any statistical differences among genotypes and N treatments (Table 3.3). Treatment differences were more evident at the R8* stage, averaging 220 kg N ha^{-1} . Genotype 3 presented 7% greater plant N content than the rest of the genotypes. Nitrogen fertilization treatments (Late- and Full-N) resulted in a 10% greater plant N content relative to the control. These results relate to the strong linear relationship between seed yield and plant N

uptake (Salvagiotti et al., 2008; Tamagno et al., 2017). The same authors documented 12.5 and 13.0 kg of seed yield increase per each kg of additional plant N uptake, in the current study the estimation was of 12 kg of seed yield per kg increase of plant N uptake at harvest.

At the R5.5 sampling, the plant fraction presenting the largest N content was the leaf ($\sim 80 \text{ kg N ha}^{-1}$), while at the R8* sampling was the seed fraction ($\sim 150 \text{ kg N ha}^{-1}$) (Table 3.3). The Zero-N treatment presented the lower N content for the total accumulation and for the stem and seed fractions, relative to the fertilized treatments at the R8* stage. Nitrogen concentration for all plant fractions at the R5.5 stage followed a decreasing order from high to low: seed ($6.3 \text{ g } 100 \text{ g}^{-1}$), leaf ($4.5 \text{ g } 100 \text{ g}^{-1}$), pod wall ($2.6 \text{ g } 100 \text{ g}^{-1}$), and stem ($1.8 \text{ g } 100 \text{ g}^{-1}$) (Table 3.4). Similar order was recorded at the R8* stage but with N concentration largely decreasing in all vegetative organs, except for the seed: leaf ($1.4 \text{ g } 100 \text{ g}^{-1}$), pod wall ($1.0 \text{ g } 100 \text{ g}^{-1}$), stem ($1.0 \text{ g } 100 \text{ g}^{-1}$), and seed ($6.6 \text{ g } 100 \text{ g}^{-1}$). Full-N treatment significantly increased N concentration in stem, leaf, and seed at R5.5; and stem and seed at the R8* stage as compared to the Zero-N and the Late-N conditions.

The decrease on N concentration and consequently on N content among all vegetative fractions (stems, leaves, and pod walls) is commonly referred as the plant N self-destructive process (Sinclair and de Wit, 1975). When seed N demand is not met due to a decrease in N uptake and N fixation rate (Thibodeau and Jaworski, 1975) the plant starts remobilizing all N resources to the sink (seeds) to meet this deficit (Borst and Thatcher, 1931; Sinclair and de Wit, 1975; 1976). Greater N reductions were documented for pod walls and leaves (Figure 3.2 B). A greenhouse experiment estimated that the N concentration in abscised material at R8* can drop as low as $1 \text{ g } 100 \text{ g}^{-1}$ in leaves (compared to $1.4 \text{ g } 100 \text{ g}^{-1}$ in the current study), $0.5 \text{ g } 100 \text{ g}^{-1}$ in stems (compared to $1.0 \text{ g } 100 \text{ g}^{-1}$), and 0.5 in pod walls (compared to $1.0 \text{ g } 100 \text{ g}^{-1}$) (Streeter,

1978) (Figure 3.2 C). In agreement with the cited study, the leaf was the plant organ with greater residual N at the end of the season (R8*). Notwithstanding the large N depletion observed in the vegetative organs, based on Streeter (1978) it can be concluded that not all N in the vegetative plant fractions (stem, leaf, and pod wall) was remobilized (overall of 58 kg ha⁻¹ left as residual N).

In other major crops such as sorghum and corn, the main plant organ reservoir for remobilization and N source to the seed have been firstly attributed to the stem (Ciampitti and Vyn, 2013; Ciampitti and Prasad, 2016; Van Oosterom et al., 2010) and secondarily to leaves. A last descriptive analysis was conducted for contrasting quartiles of N content (25th and 75th) at the R5.5 stage, for identifying the main N sources to be potentially remobilized during the seed filling. Leaves acted as the main reservoir of N in the two conditions (33.9 and 134.9 kg N ha⁻¹, 45 and 51% of redistributable N), this result was clearly connected to its high and stable N concentration (4.5 and 4.6 g 100 g seed⁻¹), and high levels of biomass production (760 and 2911 kg ha⁻¹) (Table 3.5).

Nitrogen dynamics: Remobilized N and N gain between R5.5 and R8*

Nitrogen in the seed at harvest was estimated as the sum of remobilized N during the seed filling, N present at the R5.5 sampling, and the N gained from the R5.5 to the R8* stage (Figure 3.1). Remobilization was the main N source for the seed fraction at harvest, vegetative plant organs and pod walls contributed to 95 kg N ha⁻¹, representing 59% of seed N content at R8* (Table 3.3). Similarly, several authors studying soybean N remobilization (Egli et al., 1983; Kumudini et al., 2002; Sadras and Egli, 2008; Zeiher et al., 1982) remarked the importance of this process to satisfy seed N demand. In this study, the main N remobilization organ was the

leaf, accounting for 52% of the total remobilized N, followed by pod walls, 30%, and the stem, 18% (Figure 3.2 A). A compendium of scientific literature also presented the leaf organ as the main source for seed N in soybeans (Dalling et al., 1976; Derman et al., 1978; 1983; Lewis and Pate, 1973; Zeiher et al., 1982). Soybean was found to have larger N losses in leaves as compared with other legumes (pigeon pea, *Cajanus cajan L.*; and peanut, *Arachis hypogaea L.*), and thus increasing the vegetative N contribution to the soybean seed (Devries et al., 1989). In agreement with Egli et al. (1978), pod wall and stem plant fractions were a secondary N source for satisfying N seed demand. Evidently, N remobilization is one of the most important contributors of seed N but the N gain occurring between R5.5 and R8* stages also plays a critical role for satisfying soybean N demand. On overall, 18 kg N ha⁻¹ were gained during the seed filling (Figure 3.1). This source of N represented 11% of N in the seed at harvest.

The relationship between vegetative N content and N remobilization has been reviewed and summarized in corn (Pan et al., 1986; Gallais and Coque, 2005; Ciampitti and Vyn, 2013), sorghum (Ciampitti and Prasad, 2016), and wheat (Kichey et al., 2007; Bogard et al., 2010). Likewise, in the present study, a similar analysis comparing the relationship between vegetative N content at R5.5 stage and N remobilization portrayed a strong positive relationship ($R^2 = 0.90$; Figure 3.3) regardless of the genotype and N treatments (Table 3.3). The slope of the association indicated that 61% of vegetative N at R5.5 was remobilized to the seed before harvest. Similar findings were previously synthesized in corn (Ciampitti and Vyn, 2013) with an associated slope of 60%, 55% in sorghum (Ciampitti and Prasad, 2016) and 83% in wheat (Kichey et al., 2007). Thus far, only the association reported for wheat resulted in greater N remobilization efficiency from non-seed tissues to the seed at harvest. The N reservoir before seed filling period was

related to the size of the plant, meaning that the greater the plant biomass level at R5.5, the greater the quantity of N remobilized (bubble sizes; Figure 3.3).

Negative relationships between remobilized N and reproductive N uptake are well documented in the literature for corn (Pan et al., 1986; Gallais and Coque, 2005; Ciampitti and Vyn, 2013), sorghum (Ciampitti and Prasad, 2016), and wheat (Kichey et al., 2007; Bogard et al., 2010), even when at different growth stages, i.e. flowering to maturity. Other studies have conducted similar analysis for N remobilization in soybeans focusing in cultivar differences (i.e., maturity group and growth habit) (Zeihner et al., 1982) (Kumudini et al., 2002); and drought stress on different developmental stages (Egli et al., 1983) but not really exploring what happened with the net gain of N as compared to remobilized N. In the current study, analyzing contrasting genotypes and N fertility conditions, N gained between the R5.5 and R8* stages explained 29% of changes in remobilized N (Figure 3.4 A). A negative relationship was observed between these plant N sources, implying a low N remobilization when N gain increases. This trend followed the plant growth rate experienced during the R5.5 to R8* period, larger N gain as biomass increases. Similar trade-off were observed in corn (Ciampitti and Vyn, 2013) and sorghum (Ciampitti and Prasad, 2016). Both studies documented that this relationship was less consistent ($R^2 = 0.17$ for corn and $R^2 = 0.13$ for sorghum) relative to soybeans (Figure 3.4 A, $R^2 = 0.29$). The negative slopes of the remobilized N and N gain associations reported in corn and sorghum (39 and 48%, respectively) showed that remobilized N penalized the gain of new N in a lesser extent as compared to soybean (Figure 3.4 A, 54%). To expand this understanding, the residuals from the remobilized N versus the N gained relationship were plotted against seed yield (Figure 3.4 B). The residuals followed a significant positive trend ($P < 0.05$; $R^2 = 0.57$), with positive residuals for superior yield while negative for low yield values.

This means that larger residuals happened as yield potential increases, and thus, greater is the uncertainty of the N source.

Notwithstanding, there were no statistical differences given by genotype and N treatments on remobilized N and N gained from the R5.5 to the R8* stage (Table 3.3), a descriptive analysis was conducted to identify any trends in the dependency of these two N sources for addressing seed N demand at harvest. As a first step, by genotype factor, the analysis showed that the older soybean genotypes (genotype 1, non-RR) depended in a greater proportion on remobilized N (64%) as compared with the modern genotypes (genotype 3, RR-2), with 51%, while the modern genotypes showed a larger dependency (four-fold increase, 19%) on the N gained during the seed filling relative to the older genotype (5%) (Table 3.5). The remobilized N to N gain ratio, where the lower the ratio the greater the dependency of remobilized N for supplying N to the seed, was as high as 13 for genotype 1 and as low as 2.7 for genotype 3.

An analysis of the low (25th quartile) and high (75th quartile) of seed yield distribution was performed. “Low yields” (<1600 kg ha⁻¹, 25th quartile) were mostly dependent on N remobilization (85%) to meet seed N demand. On the other hand, “high yields” (>3400 kg ha⁻¹, 75th quartile) decreased N remobilization dependency to 66%, with N gain process during the seed filling increasing from 0% to 8% (Table 3.6). Nitrogen remaining in vegetative tissues (stem, leaf, and pod wall) as potential (based on minimum N concentrations presented in Streeter, 1978) source for remobilization to the seeds at harvest was in overall 24 kg ha⁻¹, which could have represented a 15% increase of N in the seed.

Conclusions

Nitrogen remobilization and N gain during the seed filling was neither affected by the genotypes nor by the N treatments. The total plant N content at R5.5, i.e. the vegetative N reservoir, was related to plants size and dictated the potential remobilized N during the seed filling period. A trade-off between N remobilization and N gain during the seed filling was documented in this study. On overall, N remobilization supplied 59% of the seed N demand, primarily contributed by the leaf fraction, 52%. Net N gain during the seed filling represented only 11% of the seed N demand at R8*. Lastly, low yields were mainly dependent on the N remobilization process, while greater yields were dependent on both, N remobilization (still in a greater proportion) plus the N gain during the seed filling for satisfying seed N demand in soybean. The overall N remobilization primarily satisfied seed N demand via contribution of leaves, pod walls, and stems in order of priority.

Future research lines should investigate the effect of biological N fixation and its interaction in satisfying seed N demand and the interplay with N uptake and N remobilization during the seed filling period for soybeans.

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Figures

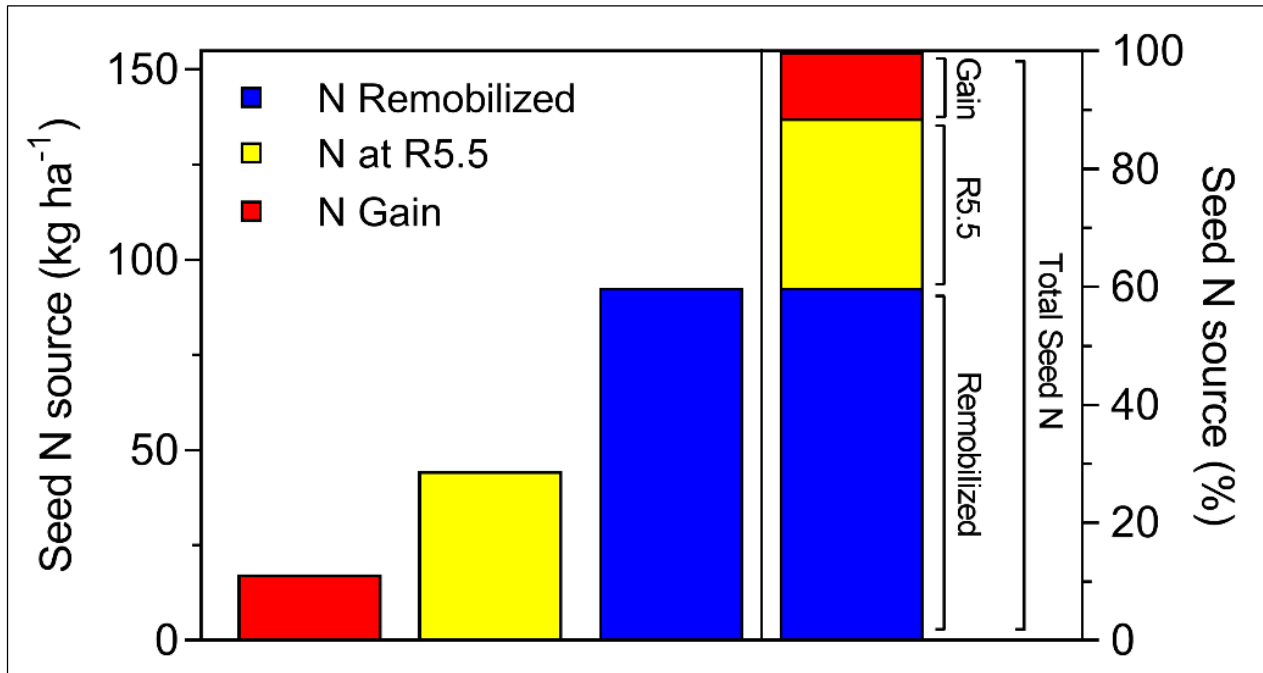


Figure 3.1 Overall seed nitrogen sources for three genotypes and three N conditions during the 2016 and 2017 growing seasons in Kansas, US. Calculations for N remobilized (Eq. 2) and N gain (Eq. 5) can be found in the materials and methods section. Nitrogen at R5.5 is the N observed in the seed at the first sampling time (Table 3.3).

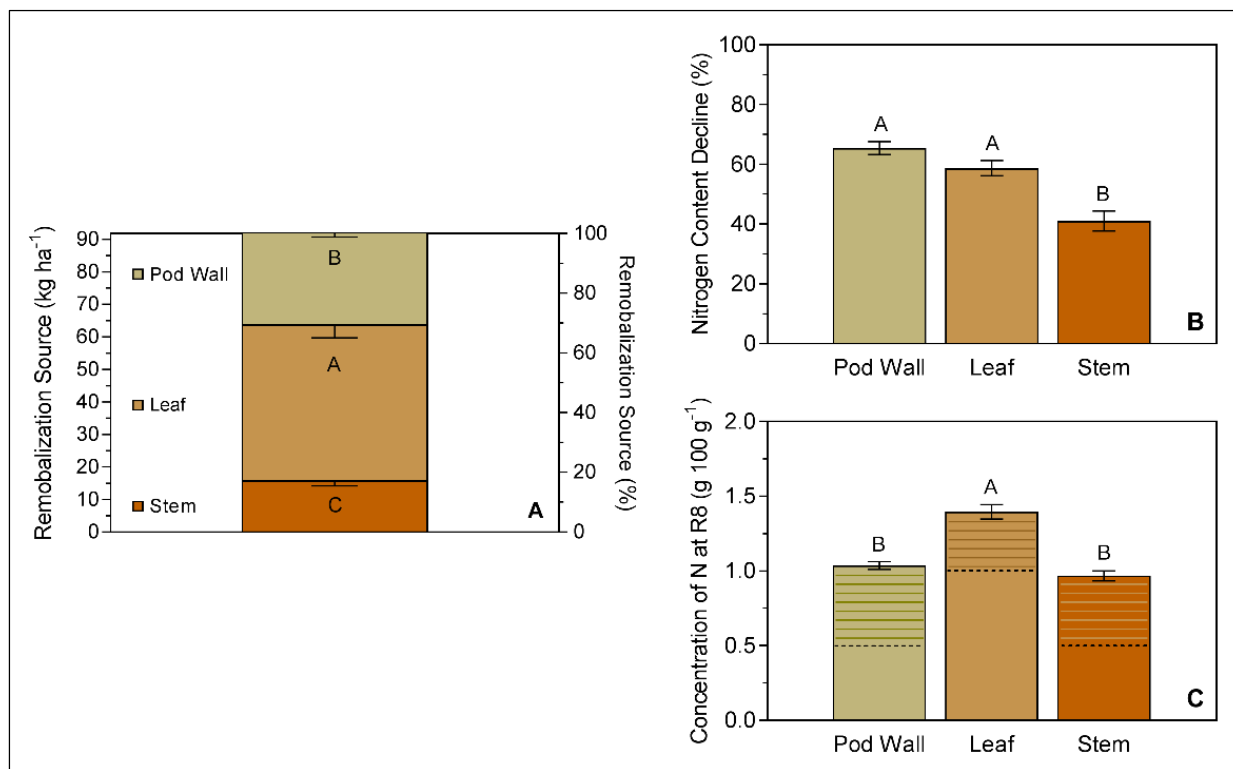


Figure 3.2 Nitrogen remobilization for each plant organ and total N gain from R5.5 to R8* growth stage, as an average across all treatments (A); N depletion, expressed in relative terms, calculated as the N content at R8* relative to R5.5 (B); and final residual N concentration at R8* stage compared to minimum N concentrations for each plant fraction observed in Streeter (1978), area above dashed lines illustrate the residual N not redistributed based on minimum levels reported. Error bars show the standard error of the mean. Different letters represent the least significant difference (LSD) at $P < 0.05$.

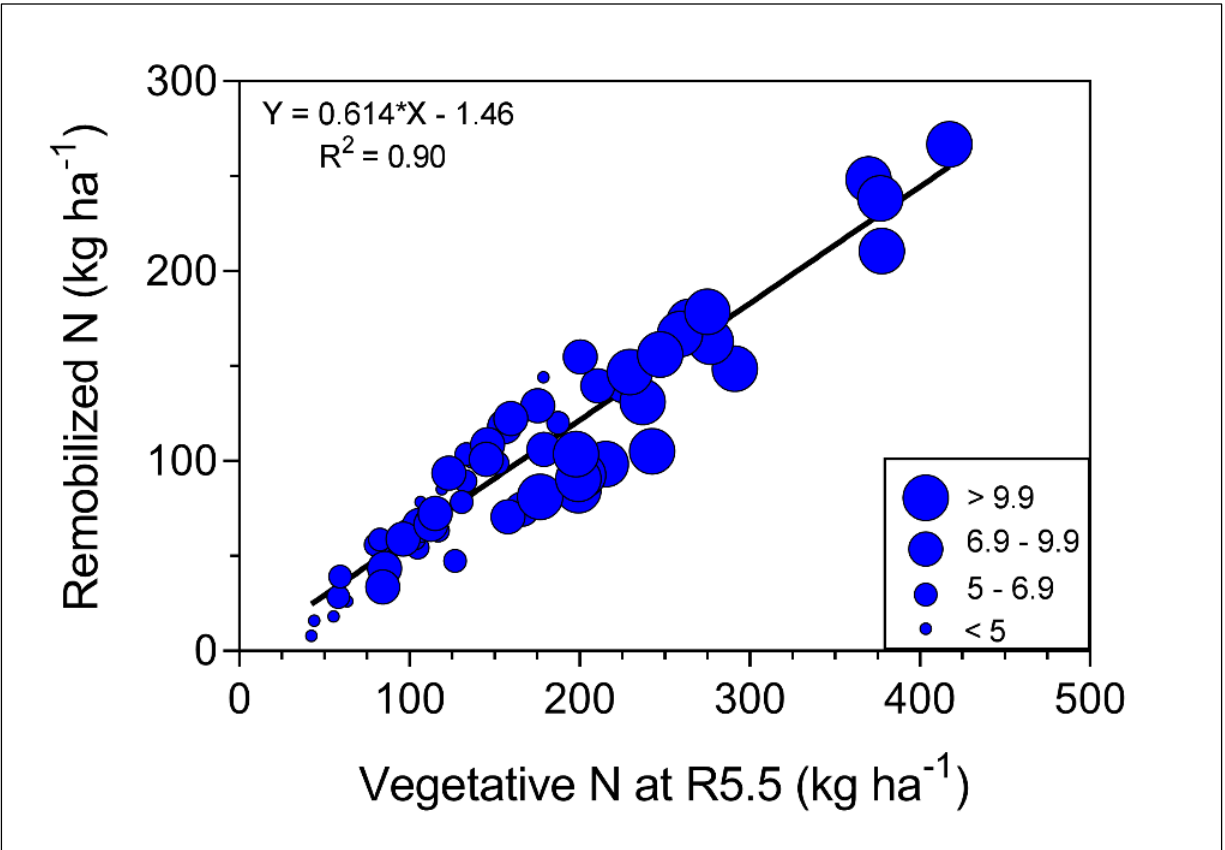


Figure 3.3 Relationship between Remobilized N (from R5.5 to the R8* growth stages) and the Vegetative (stem + leaf + pod wall) N content. Different bubble sizes represent the total plant biomass, expressed in dry basis, accumulated at R5.5 stage.

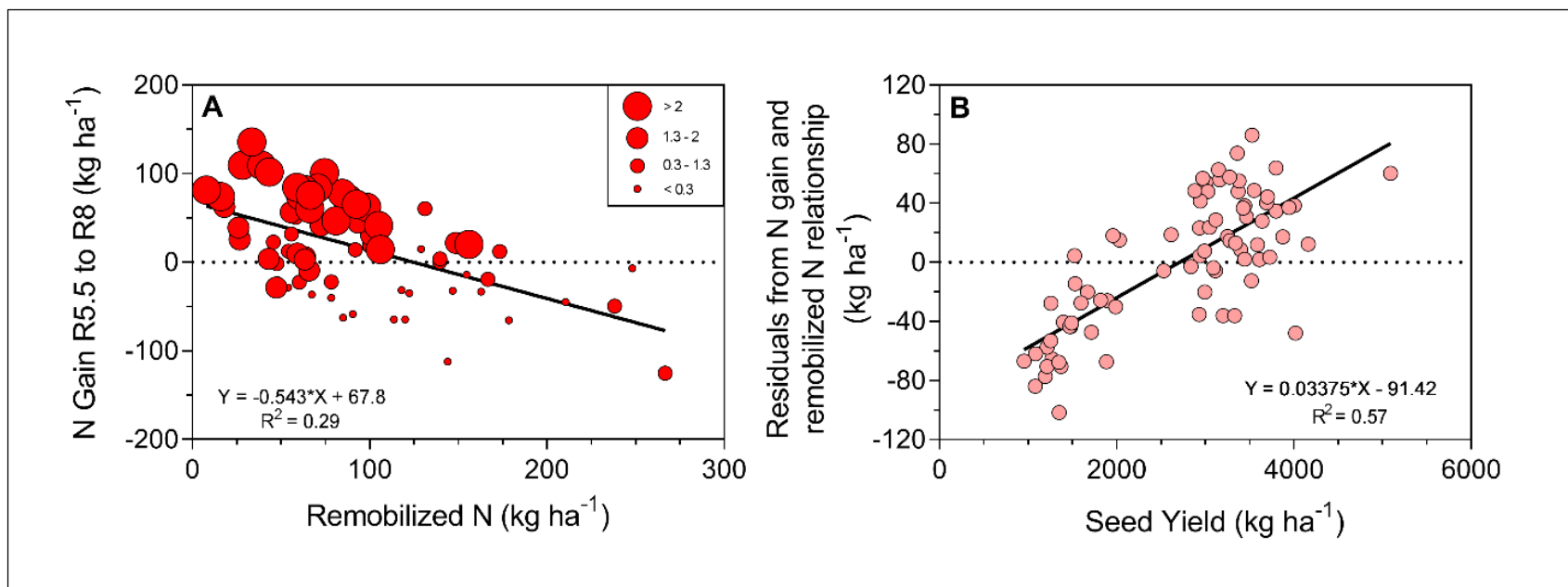


Figure 3.4 Relationship of N gain (between the R5.5 to the R8* growth stages) and Remobilized N. Different bubble sizes represent the biomass gain from R5.5 to R8* growth stages (A); and the residuals from the relationship in panel A, plotted against the seed yield, expressed in 0.13 g kg⁻¹ seed moisture content (B).

Tables

Table 3.1 Soil and climate characterization for 2015 and 2016 growing season in Kansas (US).

Year	Location	Coordinates	Precipitation (mm)	T Max (°C)	T Min (°C)	Soil Type Series	Soil pH	CEC meq/100 g	OM (%)	N-NO ₃ ppm	Mehlich P ppm	K ppm	Ca ppm	Mg ppm
2015	Ashland	39° 8' N; 96°37' W	760	35	7	Crete	7.9	13.2	1.6	2.5	60	264	2145	71
	Ottawa	38°32' N; 95°14' W	660	32	4	Woodson	6.5	25.9	3.3	-	7	191	3273	532
2016	Ottawa		533	31	8	Woodson	5.7	18.5	4.3	5	14	80	2665	393

Table 3.2 Total plant biomass and in different fractions (stem, leaf, pod wall, seed) at the R5.5 and R8 stages, seed yield (13% moisture), seed number, seed weight, and harvest index (HI) for all genotypes and N rates.

Genotypes	N Rates	Biomass at R5.5 Stage					Biomass at R8 Stage					Seed Yield	Seed Number	Seed Weight	HI
		Stem	Leaf	Pod Wall	Seed	Total	Stem	Leaf	Pod Wall	Seed	Total				
		kg ha ⁻¹										kg ha ⁻¹	# seed m ⁻²	mg seed ⁻¹	
Genotype 1		2362	1679	1566	663	6358	1998	1856	1186	2329 B [†]	7276 B	2684 B	1778 B	151 A	0.32
Genotype 2		2297	1710	1680	725	6339	2027	1951	1164	2286 B	7522 AB	2628 B	1877 B	140 A	0.31
Genotype 3		2229	1601	1621	758	6235	2158	1928	1259	2553 A	7917 A	2923 A	2103 A	139 B	0.33
	Zero-N	2285	1571	1508	697	6060	1936 b	1746 b	1125 b	2242 b	7034 b	2574 b	1788 b	144	0.32
	Late-N	2338	1758	1756	723	6622	2159 a	2074 a	1257 a	2464 a	8019 a	2826 a	2019 a	140	0.31
	Full-N	2264	1662	1602	726	6249	2086 ab	1914 ab	1228 a	2464 a	7662 a	2834 a	1941 ab	146	0.33
Genotypes		ns	ns	ns	ns	ns	ns	ns	ns	***	*	***	***	***	ns
N Rates		ns	ns	ns	ns	ns	**	*	**	**	***	**	**	ns	ns
Genotype x N Rates		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability level, respectively. ns: nonsignificant.

[†] Different letter represents the least significant difference (LSD) at P < 0.05. Capital case letters used for genotype differences, and lower case for N rates.

Table 3.3 Total N content in plant and fractions (stem, leaf, pod wall, seed) in the R5.5 and R8 stages, remobilized N, and N gain for all genotypes and N rates.

Genotypes	N Rates	Nitrogen Content at R5.5 Stage					Nitrogen Content at R8 Stage					Remobilized N	Nitrogen Gain R5.5 to R8*
		Stem	Leaf	Pod Wall	Seed	Total	Stem	Leaf	Pod Wall	Seed	Total		
kg ha ⁻¹												kg ha ⁻¹	
Genotype 1		42	76	41	42	204	22	25	13	154 B [†]	211 B	99	7
Genotype 2		38	79	45	42	201	23	27	13	151 B	217 AB	99	15
Genotype 3		37	72	39	50	198	24	26	13	167 A	229 A	85	32
	Zero-N	36	68	39	42	185	22 b	23	12	146 b	203 b	86	18
	Late-N	39	80	46	45	209	23 a	27	13	159 a	223 a	101	14
	Full-N	42	78	41	48	209	24 a	27	14	166 a	231 a	96	21
Genotypes		ns	ns	ns	ns	ns	ns	ns	ns	**	*	ns	ns
N Rates		ns	ns	ns	ns	ns	**	ns	ns	***	***	ns	ns
Genotype x N Rates		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability level respectively. ns: nonsignificant.

[†] Different letter represents the least significant difference (LSD) at P < 0.05. Capital case letters used for genotype differences, and lower case for N rates.

Table 3.4 Nitrogen concentration in plant fractions (stem, leaf, pod wall, seed) at the R5.5 and R8 stages for all genotypes and N rates.

Genotypes	N Rates	Nitrogen Concentration at the R5.5 Stage				Nitrogen Concentration at the R8 Stage			
		Stem	Leaf	Pod Wall	Seed	Stem	Leaf	Pod Wall	Seed
g 100 g ⁻¹									
Genotype 1		1.94	4.52	2.61 A [†]	6.31	0.97	1.44	0.99 B	6.62
Genotype 2		1.77	4.60	2.65 A	6.22	0.99	1.40	1.11 A	6.58
Genotype 3		1.79	4.46	2.38 B	6.23	0.96	1.32	1.01 AB	6.52
	Zero-N	1.65 b	4.38 b	2.56	6.13 b	0.95 b	1.39	1.03	6.53 b
	Late-N	1.72 b	4.49 b	2.58	6.23 b	0.94 b	1.31	1.03	6.47 b
	Full-N	2.12 a	4.70 a	2.50	6.40 a	1.02 a	1.46	1.04	6.72 a
Genotypes		ns	ns	***	ns	ns	ns	*	ns
N Rates		***	***	ns	***	**	ns	ns	***
Genotype x N Rates		ns	ns	ns	ns	**	ns	ns	ns

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability level respectively. ns: nonsignificant.

[†] Different letter represents the least significant difference (LSD) at P < 0.05. Capital case letters used for genotype differences, and lower case for N rates.

Table 3.5 Summary statistics (overall means) for biomass, N concentration, and N content levels (25th and 75th percentiles of N content) at the R5.5 stage for 2015 and 2016 growing seasons in Kansas (US).

Quantile	N in	N in	N in	N in	Stem	Leaf	Pod wall	Seed	Total	Stem	Leaf	Pod wall	Seed	Total
	Stem	Leaf	Pod wall	Seed	biomass	biomass	biomass	biomass	biomass	N content	N content	N content	N content	N content
	g 100 g ⁻¹				kg ha ⁻¹									
Low, 25th	1.9	4.5	2.6	6.4	863	760	988	444	3056	15.7	33.9	26.0	28.1	103.7
High, 75th	1.6	4.6	2.5	6.1	4331	2911	2378	916	10537	68.2	134.9	59.5	56.2	318.7
Ratio, Q75/Q25	0.8	1.0	1.0	1.0	5.0	3.8	2.4	2.1	3.4	4.3	4.0	2.3	2.0	3.1

Table 3.6 Summary statistics (overall means) of three genotypes and two yield levels (25th and 75th percentiles of seed yield) for total N content and seed N at the R5.5 and R8 stages, total N gain (R8 - R5.5), remobilized N, and non-mobilized N (residual vegetative N as potential redistributable based on minimum concentrations of N presented in Streeter, 1978) for 2015 and 2016 growing seasons in Kansas (US).

Factor	Description	Total N at R5.5	Total N at R8*	Seed N at R5.5	Seed N at R8*	Total N Gain R8 - R5.5	Remobilized N	Non-Mobilized N	N Gain Relative to Seed N at R8*	Remobilized N Relative to Seed N at R8*	Non-Mobilized N Relative to Seed N at R8	Remobilized N / N Gain
						kg ha ⁻¹						
Genotypes	1) P39B82, 1997	204	211	42	154	7	99	23.4	5	64	15.2	13.0
	2) 93Y92, 2009	201	217	42	151	15	99	24.8	10	66	16.4	6.6
	3) P34T43R2, 2014	198	229	50	167	32	85	22.5	19	51	13.5	2.7
Seed Yield	Low, 25th percentile	135	114	32	75	0	64	17.1	0	85	22.5	0
	High, 75th percentile	284	302	54	213	18	140	36.5	8	66	17	8.25

Chapter 4 - Conclusions and Future Implications

Conclusions

Chapter one investigated the current status of food production from a broad perspective on agricultural needs, and then narrowed down to specific soybean needs. Increase in global production needs are mainly due to increases in population, shifts in diets, and increases in the uses of biofuel. To date, food security is still one of the largest concerns around the globe. Major and fundamental solutions are needed to address these issues. Such proposed solutions include, but are not limited to, increase efficiency in the use of resources and productivity levels of farming systems across the globe. Soybean is among the top major crops contributing to food security as one of the largest sources of plant protein, oil, and biofuel. Although, yield improvements have been observed over a large period of time (90+ years), projections showed that future yield gain over time will not match food demand. Many research studies have contributed via recommending strategies for continuing yield improvement, even though, it is quite of a challenge for soybean due to its seed composition and large N demand. To improve soybean yield, understanding N dynamics and demand is indeed a baseline of attention for increasing productivity. In such a sense, understanding main plant changes, N sources, N demand, and N partition processes have an important role for increasing the scientific knowledge and advance the understanding of the soybean production systems around the world.

Globally, collective, and cooperative action is needed from all different sectors of society, to further advance agriculture, increase input-use efficiency of finite resources, and optimize productivity for attaining the complex goal of feeding the growing population while minimizing the environmental farming footprint.

Chapter two studied two different agricultural and pioneering countries and environments in the production of field crops: The United States and Argentina. The overall research question aimed to answer if a N limitation in soybean productivity exists, considering diverse N scenarios with historical and modern genotypes. Seed yield increased in both locations due to a combined effect of genetic improvement (from 1980s to the 2010s eras), and the addition of high rate of N (non-limiting), suggesting that N limitation exists. Beyond that, an interaction effect was also observed and suggested different levels of response between genotypes. From the interaction, productivity increases in response to the addition of high N rate were mostly observed with the modern genotypes (2000s and 2010s), connected to superior yields and greater N demand. Along with increases in seed yield, N removal in seeds was incremented, although seed protein concentration decreased when comparing historical to modern soybean genotypes. For achieving such increases in seed yield and seed N removal, the plant implemented three main strategies: i) increasing biomass, ii) producing more seeds per unit of area, and iii) improving seed size, greater individual seed weight. However, two of these plant strategies differed between the genotypic and N conditions. Genetic changes primarily impacted the number of seeds produced, while the addition of N presented a consistent positive impact on the individual seed weight. Moreover, when comparing low- versus high-yields, differences in seed number, seed weight, and seed protein were documented. The two locations and different yielding conditions were also examples that plant strategies dynamically change when targeting different levels of productivity. Those strategies were not related to the year of release of materials, but to the final productivity level attained by the soybean plants in those systems.

Chapter three focused on the main N sources and demand of harvested seeds during the critical period of yield formation in soybean (i.e. the seed filling). The results showed that N content and concentration among plant fractions at different stages are a function of the existent conditions (i.e. genotypes or N). On overall, and as for other crops (i.e. corn, sorghum, wheat), remobilization of N from vegetative organs to seeds is one of the main N sources for the N in seeds at harvest (59% of total seed N). The level of N remobilization is mainly related to the amount of N accumulated in the plant until the onset of the seed filling, without much effect of genotype or N conditions. From the different plant organs, leaves are the main N reservoir in soybean (accounting for 52% of total remobilized N). Greater amounts of remobilized N were associated with larger biomass production (until the onset of the seed filling). In addition to the N remobilization process, the new N uptake during the seed filling period is a N source for satisfying seed N demand, but in a lesser extent (11% of total seed N). Again, the magnitude of new N uptake was related to the biomass accumulation and plant size, without a direct effect of genotype or N applications. Although these are two important sources for supplying seed N, managing those become a challenge because of their trade-off (as in other crops), meaning that when N remobilization increased, new N uptake decreased. This is a process auto-regulated within the plant, and such regulations change at varying yield levels. Lower yields (with lower N demand) are mainly dependent on the N remobilization process, while on the other hand, greater yields (greater N demand) are dependent on both, N remobilization plus new N uptake processes.

Future Implications

Future research efforts should focus on improving the understanding of efficient management systems that permit equally increases in productivity and quality for soybean. For achieving this, knowledge in N dynamics and sources during the critical period of seed filling in soybean plant is needed. Understanding the interaction and dynamics of N in the soil, N fixation, and N in the plant for fulfilling seed N demand is of critical importance.

On chapter two, seed yield increases were observed over time. Those increases were accompanied with larger amounts of N due to the increased N demand. High-yielding conditions were limited by N, meaning that N from soil and or N from fixation were not enough to satisfy plant N demand. However, questions about N limitation and the way to increase the availability of N still need to be investigated. For instance, a descriptive characterization of the BNF process throughout the crop season accompanied by a characterization of the soil N supply (mineralization process) will be relevant to understand the offer-demand N dynamic for the soybean farming systems. Dissecting and understanding these two N sources will provide a solid foundation to the overall N process for this crop. Complete characterization of environments to understand “why, where, and when” N supply from soil + fixation is limited is important. Monitoring and improving factors that can affect N fixation (i.e. soil pH, soil drainage, soil compaction) are steps to start. Farther than that, increasing the efficiency of fixation (i.e. using more efficient bacteria strains) is research that should be explored in the near future. Recently, some studies claimed yield increases in response to more than one timing of inoculation during the crop growing season, suggesting increased N fixation. Study of feasible, practical, and inexpensive ways for predicting changes in patterns of BNF and estimating total contribution of N fixation at field- and research-scales will add benefits as well by speeding up the assimilation

processes. Finally, if inorganic N and or N fertilizer are to be supplemented, studying ways to reduce the tradeoff of N in soil with the N fixation process (i.e. subsoil applications, deep N placement) and increasing efficiency of applications (i.e. timing, slow release sources) will add quantitative progress and benefits to the system as a whole.

The study of seed N sources and demand presented in chapter three, introduced concepts related to changes on the N status from beginning to end of seed filling along with its implications. However, a more detailed characterization during the seed filling period, i.e. multiple sampling time, would provide more insightful representation of the temporal N dynamic. In addition, it would be of importance to better understand and dissect the sources for N on the seed, either from soil or from fixation. Such quantifications can be achieved, as presented in other crops, with the use of labelled ^{15}N techniques. Beyond this characterization, the contribution of N from roots it is also a critical piece for improving the understanding on the N dynamic, but this type of analysis is limited to the complexity of the sampling and root characterization. As it is shown in the results section, different strategies within the plant exist in terms of achieving yields and N demand, so exploring these behaviors under contrasting conditions (i.e. environments, genetics, fertility) will be still needed.