

Biological nitrogen fixation, plant nitrogen demand, and physiological processes involved in
seed yield and seed composition in soybeans

by

Santiago Tamagno

B.S., National University of Rosario, 2013

M.S., National University of Rosario, 2015

AN ABSTRACT OF A DISSERTATION

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Department of Agronomy
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

Soybean [*Glycine max* (L.) Merr.] plants can capture atmospheric nitrogen (N) through symbiotic relationships with soil bacteria in a process called “biological N fixation” (BNF), reducing the reliance on synthetic fertilizers. To meet growth of the global population and increasing demand for quality protein and oil, the need for soybean will inexorably grow. One of the challenges to further improve soybean seed yield is the high demand of N in comparison to cereals and other oilseed crops. Further characterization of the environmental and management effects on BNF process is needed to enhance its capacity for supplying N to the crop. This dissertation is organized in four chapters outlining these topics through a series of experiments describing the association between plant nutrition, BNF, and seed composition with seed yield generation. Chapter 1 presents a synthesis analysis on a nutrient-based physiological framework on four key variables determining N efficiency in soybean (i.e., seed yield, N seed concentration, total N uptake, and nitrogen harvest index). Results showed that N partitioning to the seeds is the main parameter responsible in seed yield variation instead of N seed concentration. An experiment in Chapter 2 explored the hypothesis of the carbon (C) cost of BNF on biomass production, yield formation, and seed composition. Chapters 3 and 4 explored the historical component behind seed yield generation and seed composition. Results from Chapter 3 describe the shift in allocation of reproductive biomass using the allometric approach, whereas Chapter 4 provides a detailed description of the dynamics of oil and fatty acid accumulation under two N regimes.

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Approved by:

Major Professor
Ignacio A. Ciampitti

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Dedication

To María, Desiderio, Dora, and Vladimir.

Chapter 1 - Introduction

Prospects for crop improvement in yield revolves around the current effects of climate change and the increasing global demand for grain crops. In the following years, agricultural production faces the challenges of global food security while mitigating the impact of farming systems to preserve environmental resources. In this context, improving crop yields rather than incorporating more arable land, is the path towards sustainable food production (Tilman et al., 2011; Foley et al., 2011). Current rates of improvement have been reported to be below the expected increase to meet the projected demand in the following years (Ray et al., 2013).

The contribution of legumes to agricultural sustainability is many-fold. These crops can capture atmospheric nitrogen (N) through symbiotic relationships with soil bacteria in a process called “biological N fixation” (BNF), reducing the reliance on synthetic fertilizers. To meet growth of the world’s population and increasing demand for quality protein, soybean demand will inexorably grow. Soybean [*Glycine max* (L.) Merr.] is the crop among legumes with a unique seed composition, high in oil (21%) and protein concentration (40%; Medic et al., 2014). These two aspects highlight the potential contributions to sustainability by incorporating fixed N into production systems and meeting the needs of growing world population.

One of the challenges to further improve soybean seed yield is the high demand for N (due to the high protein concentration) relative to cereals and other oilseed crops (Sinclair and de Wit, 1975). At the plant level, seed composition has implications on maternal plant physiology in either carbon (C) and N economy. Soil N supply and BNF are the two primary sources of N for the plant, with the latter contributing up to 95% (Salvagiotti et al., 2008). In exchange for N assimilation, bacteria will require photoassimilates from the host plant as a source of energy. Nitrogen is stored in vegetative organs (i.e., mainly leaves) during early vegetative stages of the

plant to be remobilized into the seeds during the seed filling period (Ortez et al., 2019).

Developing seeds depends not only on the current C and N assimilated but also on the remobilization from vegetative parts. The process of N remobilization induces leaf senescence interfering with photosynthesis and C availability for seeds and BNF, which peaks in the onset of seed filling to decay later as seeds are a stronger C sink. This sequence is how seed composition determines physiological processes in the maternal plant determinant to understand C and N correlation in this period.

The ability to fix atmospheric N does not make soybean plants independent of other sources of N, even when BNF is fully effective. When soil N is available in large amounts, BNF is reduced. Although the reasons relying on this effect are still unknown and complex, manipulation on mineral soil N is useful to understand soybean sensitivity to changes in symbiotic activity. Further characterization of the environmental and management effects on the BNF process is needed to enhance its capacity to provide N to the crop in critical stages. However, this information is still to be obtained regarding the United States soybean productive area. This dissertation is primarily focused on N sources (i.e., soil and N fixed) in soybean and the interplay between exogenous N applications influencing seed yield and seed composition. Results presented in the following chapters provide useful insights into the relationship among the processes underpinning the accumulation of biochemical seed fractions with C and N sources.

Chapter 2 - Nutrient partitioning and stoichiometry in soybean: A synthesis-analysis

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ABSTRACT

On-farm attainable soybean yields are primarily limited by nutrient and water supply. High-yielding soybean is related to high nutrient uptake. A proposed theoretical framework underpinning yield formation includes plant nitrogen (N) uptake, N harvest index (NHI), and N seed concentration (%N_{seeds}). The objectives of this study were focused on (i) investigating the effect of NHI and %N_{seed} on yield-to-uptake relation for N, and (ii) analyzing dry mass and N partitioning and extending this analysis to phosphorous (P) and potassium (K) uptake and (iii) studying the influence of specific seed:stover ratios on the relationship of N with P, and K uptake. Metadata on yield, nutrient uptake and specific-organ nutrient concentration was summarized from experiments located in three different environments: Indiana, Kansas (both US), and Argentina (herein termed as IN, KS, and ARG, respectively). The main outcomes from this research were: 1) The yield-to-uptake relation for N was primarily explained by NHI; 2) The algebraic model proposed by Sinclair (1998), that includes each specific-organ nutrient concentration explained consistently nutrient (N, P or K) harvest index (HI) as a function of HI with different trend; 3) plant nutrient ratios were primarily governed by vegetative nutrient concentration (stover fraction), acting as a nutrient reservoir or supply depending on the demand of nutrient in the seed. Further research on the nutrient and biomass partitioning should focus on

examining the HI:NHI relationship under varying genotype x environment x management interaction.

INTRODUCTION

Nitrogen (N) is essential for plant growth and seed production (Lawlor, 2002). In soybean, N demand is satisfied by biological N fixation (BNF) and by soil N supply. A historical review performed by Salvagiotti et al. (2008) documented a linear response between seed yield and plant N uptake with a 13 kg yield per unit (kg) of N taken up by the crop. Soybean N internal efficiency (slope, yield-to-plant N uptake) is smaller relative to other crops due to the high oil and protein content in seeds that demands large amounts of N uptake and remobilization from vegetative tissues (Sinclair and de Wit, 1975). A minimum N concentration (%N) in plant is needed to maintain optimum photosynthetic rate (and therefore biomass production), since most of leaf N is a constituent element of the protein ribulose 1,5-bisphosphate carboxylase involved in the photosynthesis process (Sinclair and Horie, 1989). To accomplish this demand, BNF should supply a large proportion of N during a substantial portion of the growing season (from early vegetative stages until the end of the seed-filling period).

Changes in seed yield can be understood by studying its relationship with total plant N uptake and via dissecting N uptake-related components. Total plant N uptake, N harvest index (NHI, seed N-to-plant N content ratio) and N concentration in seeds (%N_{seed}) may explain seed yield generation in crops as described in Equation 2.1:

$$\text{Seed yield (kg ha}^{-1}\text{)} = (\text{Total plant N uptake} \times \text{NHI}) / \%N_{\text{seed}} \quad \text{Equation 2.1}$$

Total plant N uptake, NHI, and %N_{seed} are all influenced by the complex genotype x environment x management practices interaction (Below, 2002; Masclaux-Daubresse and Chardon, 2011). Using the framework proposed in the Equation 1.1, Rotundo et al. (2014) reported, for a large set of genotypes evaluated in several environments, that plant N uptake was the main component in high-yielding soybean genotypes. Despite the genotypic variability in NHI and %N_{seed} found in this study, the relative influence of NHI and %N_{seed} has not been studied. Sadras (2006) found an equal contribution from both traits to seed yield and plant N uptake relationship when different legumes species were evaluated.

Equation 2.1 indicates the importance of N partitioning to seed from non-seed tissues, i.e. N harvest index (NHI) (i.e. seed N-to-plant N content ratio) (Hay, 1995) which depends on biomass partitioning to seeds in crops. i.e. harvest index (HI), seed yield-to-plant biomass (Donald and Hamblin, 1976). Increases in HI are usually positively correlated with NHI, since greater biomass accumulation in seeds results in an N adjustment in terms of partitioning between organs in the plant (Sinclair, 1998). This correlation has been also observed in other legumes such as common bean (Araújo and Teixeira, 2003) and in cereals (Ciampitti and Vyn, 2012; Löffler et al., 1984). For the same authors, the HI:NHI relationship has been studied as a linear response regardless of other plant traits. For instance, in soybean, NHI and HI may also vary as a function of seed protein concentration because of different biomass and N partitioning during the seed-filling period (Salado-Navarro et al., 1985), and also may be modified by residual %N present in the stover fraction (herein termed as %N_{stover}) and their N stoichiometry ratio (“seed:stover N stoichiometry”) with seed N concentration (herein termed as %N_{seed}). This approach has been summarized algebraically in a formula proposed by Sinclair (1998):

$$NHI = \frac{[\%N_{seed} \times HI]}{[HI \times (\%N_{seed} - \%N_{stover}) + \%N_{stover}]}$$

Equation 2.2

Lawn (1989) suggested that improvements in HI are constrained by NHI, and indirectly by both $\%N_{seed}$ and $\%N_{stover}$. This author proposed a theoretical framework for the NHI and HI relationship (Equation 2.2), suggesting a curvilinear response in crops with high $\%N_{seed}$ and low $\%N_{stover}$, and being almost linear when $\%N_{seed}$ approaches $\%N_{stover}$. The latter situation would be reflected in crops such as maize and sorghum where the N concentration in grain and stover are closer (ca. 1:2 ratio; Jones, 1983).

Both approaches explaining the NHI:HI relationship has not been documented for a specific crop species with data from field studies. Additionally, to the extent of our knowledge, this theoretical approach was only evaluated for the NHI:HI relationship but not for other macronutrients.

Nitrogen should be in balance with other macronutrients such as phosphorus (P) and potassium (K) in order to maximize seed yield. An approach for understanding these balances can be pursued by analyzing plant nutrient stoichiometric ratios (e.g., N:P and N:K ratios). For example, as biomass increases during the plant growth cycle, plant N:P ratio tended to decrease because of changes in nutrient concentrations in the plant (Greenwood et al., 2008). This occurs because of the differentiation of tissues (e.g., leaves, stem, roots) during the crop cycle, with each tissue having different proportions of nutrients according to its function (Greenwood et al., 2008). Variability in N:P and N:S ratios for legumes species and more specifically for soybean have been previously documented (Sadras, 2006; Salvagiotti et al., 2012). However, previous research studies did not consider the influence of the variation in nutrient concentration related to specific plant organs. Moreover, characterization for P and K stoichiometry in modern soybean

cultivars over a wide range of genotypes and environmental conditions has not yet been reported in literature.

The aims of this study were to 1) provide evidence about the relative effect of NHI and %N_{seed} on yield-to-N uptake relationship; 2) implement the mathematical framework proposed by Sinclair (1998) for NHI:HI relationship in soybean and extend it to P and K, and 3) study the influence of seed:stover ratios on the relationship of N with P, and K uptake.

MATERIALS AND METHODS

Experimental data

Data from experiments carried out in Argentina (herein termed as ARG) and the US (Kansas, KS, and Indiana, IN) were used, including different genotypes, environments and management practices (Table 2.1). The final database included: 1) seed yield (dry basis), 2) %N, %P and %K in seeds and stover, and 3) seed, stover, and total biomass.

Eight site-years were summarized from 2009 to 2014 seasons, comprising 167 data points (including replications) for all plant traits evaluated. At all site-years, the experimental layout was a complete randomized block (CRB) design with three (ARG, IN) and five (KS) replications. Planting dates, seeding rates and crop protection practices were in accordance with local farmer practices. Plots were between four (KS and ARG) to eight (IN) rows width and 4 to 5 m long. Row spacing differed by region with 0.52 m spacing in ARG, 0.76 and 0.38 m in KS, and 0.76 m in IN.

Crop measurements

Phenological stages were recorded during all seasons and biomass was sampled in R7 for ARG and KS, and R8 for IN (Fehr and Caviness, 1977) in representative rows of each plot. For all locations biomass sampling included leaves in standing biomass except for IN. Differences in sampling time across all environments should not affect final nutrient content since maximum nutrient content is achieved at or right before those growth stages (Bender et al., 2015).

Seed yield was measured by harvesting a representative area of each plot. Samples were weighed and adjusted to dry basis. Aboveground biomass (BM) was collected by cutting plants at the ground-level in a 1 m² area (KS and ARG) and in a 0.76 m² area (IN). Plants collected from each plot were separated in non-seed (herein term “stover”) and seed biomass fractions and dried at 65°C until constant weight was attained. Samples were ground to determine total nutrient concentration (%nutrient, N, P and K) via the combustion method for N (AOAC, 2000) in KS and IN, and Kjeldahl in ARG. For P and K concentration was made via the inductively coupled plasma (ICP) spectrometry method (AOAC, 2000).

Nutrient uptake for each plant fraction was calculated as the product of %nutrient multiplied by the specific-organ mass (dry basis). Plant nutrient uptake was calculated as the sum of stover and seed nutrient content (Equation 2.1).

$$\text{Plant Nutrient Uptake (kg ha}^{-1}\text{)} = \left[\% \text{ Nutrient}_{\text{stover}} \times \text{BM}_{\text{stover}} (\text{Kg ha}^{-1}) \right] + \left[\% \text{ Nutrient}_{\text{seed}} \times \text{BM}_{\text{seed}} (\text{Kg ha}^{-1}) \right] \quad \text{Equation 2.3}$$

Harvest index (HI) and nutrient HIs (NHI, PHI, KHI) were determined as the seed to aboveground biomass (BM) or nutrient content ratio [Equation (2.4); (2.5)].

$$HI = \frac{\text{Seed (kg ha}^{-1}\text{)}}{\text{BM (kg ha}^{-1}\text{)}} \quad \text{Equation 2.4}$$

$$\text{Nutrient HI} = \frac{\text{Seed Nutrient Uptake (kg ha}^{-1}\text{)}}{\text{Plant Nutrient Uptake (kg ha}^{-1}\text{)}} \quad \text{Equation 2.5}$$

Statistical analysis

Descriptive statistics were performed to summarize all measured variables: minimum, maximum, interquartile range, mean, and standard deviation of the mean. When differences between slopes from each data set were not significant ($p > 0.05$), a unique linear regression was performed for the relationship between seed yield-to-plant N uptake. The y-intercept (y-int) was forced to zero since the analysis without constraints was biologically unrealistic ($y\text{-int} > 0$), this extrapolation was done only for the range of data analyzed. The effect of genotype, environment (grouped in year and location), management (irrigation) and their interaction was quantified through the estimation of the variance for all variables using the VARCOMP procedure (SAS Institute, 2012) following Assefa et al. (2016).

The relationship between nutrient HI and HI has been algebraically described for N as an asymptotic response of NHI to changes in HI (Sinclair, 1998). As presented in Equation 2.2 the NHI is influenced by the specific-organ N concentrations. The approach proposed by Sinclair (1998) can be extended to P and K nutrients.

Nutrient HI was plotted against HI and a curve following Equation 2.2 was fitted using the NLIN procedure of SAS software (SAS Institute, 2012) to estimate parameters of %Nutrient_{seed} and %Nutrient_{stover} for each nutrient (N, P, and K). Parameters for the curves of the 95% confidence intervals were obtained from individual predicted values of the model from each main curve.

An exponential function $Y = B1 * XB2$ (Cassman et al., 2002) was fitted for the relationship of plant N versus P and K uptake for assessing the specific organ influence on N:P and N:K ratios. These relationships defined envelope functions (mean and maximum-minimum boundaries) and N:P and N:K ratios to show the frequency distribution as data descriptors. The non-linear regression model and analysis mentioned above were performed with GraphPad Prism software (GraphPad Prism 6; Motulsky and Christopoulos, 2003). Residuals from the plant nutrient uptake relationships (Fig. 2.4A, B) were correlated against specific-organ nutrient concentration (both seed and stover fractions). Proportion of variance (i.e., coefficient of determination, R^2 values) accounted for each nutrient plant fraction (Fig. 2.4C) was evaluated following the procedure utilized by Sadras (2006) and Ciampitti and Vyn (2013). Outlier calculation and identification was executed via the robust standard deviation of the residuals (RSDR) following the method developed by Motulsky and Brown (2006).

RESULTS

Average seed yield for pooled data was 3.4 Mg ha^{-1} (Table 2.2). Seed yield ranking from high to low was $\text{KS} > \text{ARG} > \text{IND}$ ($3.9 > 3.5 > 2.9 \text{ Mg ha}^{-1}$; data not shown). Data dispersion represented through the standard deviation (SD) was 1.2 Mg ha^{-1} and ranged between 0.8 and 7.9 Mg ha^{-1} . Aboveground plant biomass at the end of the season mimicked the trend documented for seed yield (Table 2.2) with a range of values explored from 2.0 to 15.5 Mg ha^{-1} . Mean biomass average was 8.6 Mg ha^{-1} with a SD of 2.9 Mg ha^{-1} . Average HI was 0.40 and presented similar values across data sets. Even though the range of values was large (0.19 to 0.63) data was concentrated between 0.35 and 0.45 (25% and 75% percentiles, respectively).

Yield related traits: plant nitrogen uptake, NHI and %N_{seed}

The slope of the forced y-int of the yield-to-plant N uptake relationship was utilized to study the overall model and N efficiency term (slope). The slope was 0.0125 Mg yield kg⁻¹ N (Fig. 2.1A) with all data sets fitting a unique lineal regression model. Average plant N uptake for the metadata was 267 kg N ha⁻¹ and, as observed with yield and biomass, plant N uptake presented a large range of values from 65 to 549 kg N ha⁻¹ (overall mean ranging from high to low: KS, 342; ARG, 323, and IN, 206 kg N ha⁻¹).

In this study, pooled data for NHI and %N_{seed} were poorly correlated ($r < 0.24$; data not shown). Residuals of the yield-to-plant N uptake relationship were calculated and regressed against both NHI and %N_{seed} (Figs. 2.1B, C). Residual analysis revealed that there was a positive linear relationship between the NHI and the residuals ($p < 0.01$) but not with residual and %N_{seed}. Positive residuals of the yield-to-plant N uptake reflect that soybean partitioned relatively more N to the seed (high NHI) than the average and a negative residual means soybean partitioned relatively less N to the seed (low NHI) than the average (Figs. 2.1B, C). These results suggest that the yield-to-uptake relation for N can primarily be explained by NHI.

Genotype, environment and management effects

A large proportion of the variation in the dataset was explained by the environment (i.e., 23 to 93%; Fig. 2.2). Large amplitude observed in seed yield can be attributed to the G x E x M interaction and the residuals, which accounted for 44% of the total variance. However, environment and management factor explained 29 and 24%, respectively, and only 3% was due to genotypic variation. Those results are in agreement with the range of environmental conditions explored in all experiments (Table 2.1). Plant N uptake followed a similar trend relative to seed

yield, with a substantial variation explained through the G x E x M interaction and the residuals. Nitrogen HI and %N_{seed} presented similar genotypic effect, however the proportion of interaction and residuals was lower in the latter. Also, 82% of the variation was accounted for environmental effects and just 7% for the genotype factor. This result is in agreement with data dispersion in Table 2.2, showing that most of the genotypes have intrinsically low variability in %N_{seed} (i.e. CV below 10%). For NHI, 62% of the variation was explained by the environmental conditions and a 31% was for G x E x M interaction and the residuals. Meanwhile, the genotypic effect represented 7% in a similar proportion than %N_{seed}.

Nutrient partitioning and harvest index

Mean N partitioning expressed as NHI was 0.75 for the metadata (Table 2.2). For all environments, NHI values were comparable, i.e., 0.83, 0.71, and 0.72 for IN, KS, and ARG, respectively. The NHI was plotted against HI to develop a unique curve (Fig. 2.3A). Equation 2.2 was used to represent the NHI-to-HI relationship using observations collected from all field studies, the parameters %N_{seed} and %N_{stover} for the fitted curve ($p < 0.001$; $R^2 = 0.60$) were 5.89 and 1.28 g 100 g⁻¹, respectively (Fig. 2.3A). Then, the %N_{seed}:%N_{stover} ratio was 4.6:1 for the NHI:HI, ranging from 2.5:1 to 10.7:1, considering the lower and upper boundary of the confidence intervals. When residuals of the model (NHI:HI) are related with the %N_{seed}:%N_{stover} stoichiometric ratio they explained 96% of variability, hence, differential HI and NHI responses were primarily related with changes in this ratio as postulated by Lawn (1989). The upper boundary function of the model was represented by high %N_{seed} (8.24 g 100 g⁻¹) and low %N_{stover} (0.76 g 100 g⁻¹); while the lower boundary function depicted a closer %N between seed (4.22 g 100 g⁻¹) and stover (1.71 g 100 g⁻¹) (still with %N_{seed} > %N_{stover}). Variation, expressed as ((Max –

Min)/Min)*100 (Sadras, 2007), was 92% for %N_{seeds}, ranging from 3.89 to 7.47 g 100 g⁻¹, while variation was 660% for %N_{stover}, ranging from 0.53 to 3.81 g 100 g⁻¹ (Table 2.2).

Data from P showed a similar trend when PHI and HI were modeled using Equation 2.2 (Fig. 2.3B). Fitted values for %P_{seed} and %P_{stover} parameters were 0.86 and 0.20 g 100 g⁻¹ ($p < 0.001$; $R^2=0.36$), respectively. These specific-organ %P differed by approximately 50% above the average values recorded for the overall metadata (i.e., 0.53 and 0.13 g 100 g⁻¹; Table 2.2). Although different dispersion was observed through the confidence intervals, the PHI:HI relationship showed a similar trend as that observed for the NHI:HI ratio, depicting a curvilinear response that tends to a plateau. Between each data set mean values were dissimilar when KS and IN vs. ARG are compared, the two first data sets are showing a higher mean average for PHI (0.75 and 0.84) and %P_{seed} (0.53 and 0.60 g 100 g⁻¹, respectively) compared with ARG (0.64 for PHI and 0.47 g 100 g⁻¹ for %P_{seed}).

Potassium HI depicted a different response when regressed against HI (Fig. 2.3C). The model fitted for the data ($p < 0.001$; $R^2=0.53$) tended to be more linear than NHI and PHI, i.e. close to the 1:1 ratio. The latter is primarily explained by the similarities documented in the absolute values for %K in both seed and stover fractions. Mean values for these variables were 1.70 and 1.42 g 100 g⁻¹ for %K_{seed} and %K_{stover}, respectively (Table 2.2), which were similar to the parameters estimated by the model (%K_{seed}= 1.64; %K_{stover}=1.36). Compared with NHI and PHI, the HI:KHI response function was mainly affected by the term %K_{seed} minus %K_{stover} of Equation 2.2, which for some data points this term was close to zero or negative (%K_{stover} > %K_{seed}), thus affecting the curvature of the lower boundary for the KHI:HI model. This can be depicted through the points below the 1:1 line in Fig. 2.3C. Data distribution showed a different K dynamic at low HI values (ca. <0.40) indicating K accumulation with a limitation on the sink

capacity (i.e., seeds) to be translocated; thus, the stem is acting as a reservoir organ as indicated by the high %K_{stover}. The main difference in K vs. N and P is related to the intrinsic nature of nutrient HI with K presenting an overall mean of 0.45 vs. 0.73 and 0.75 for P and N, respectively (Table 2.2).

N:P and N:K stoichiometry

The relationship between plant N and P uptake is depicted in Fig. 2.4A. A curvilinear response was the best-fit model ($R^2 = 0.68$) showing greater plant P uptake as yield increases (showed as varying bubble sizes). The N:P ratio frequency distribution was different for each data set (Fig. 2.4A.1). The most frequent N:P ratio ranged from 9.8:1 to 12.3:1 (between 25% and 75% quartiles), following the rank from low to high: ARG>IN>KS (Fig. 1.4A.1). The upper boundary of the N:P ratio was 20:1 portraying a maximum P dilution (minimum P uptake per unit of N uptake); while the lower boundary was close to 7:1 depicting a maximum P accumulation in relation with N uptake. Mean N:P ratio values were 10.4, 10.5, and 13.3 units for IN, ARG, and KS, respectively (Fig 2.4A.1).

Moreover, the interaction between N and P uptake was influenced by changes in %P in different plant organs (i.e. stover and seed). The latter was accounted for by analyzing residuals of the plant N-to-P uptake regression for all data sets. A large proportion of the variation on plant N-to-P uptake was accounted for by changes in %P_{seed} and %P_{stover} ($p < 0.001$; Fig. 2.4C) but varying for each fraction. This is reflected though variation in residuals which was primarily explained by changes in %P_{stover} ($R_2 = 0.38$); while changes in %P_{seed} accounted for less variation ($R^2 = 0.05$) (Fig. 2.4C).

A curvilinear model was fitted for the plant K and N uptake ($R^2 = 0.74$; Fig. 2.4B) with a positive response (parameter $B2 < 1$) and same as model fitted for P uptake, high yield (expressed through the bubble sizes) were associated with high nutrient uptake across all regions. A broader N:K range was documented for ARG (min = 1.4; max = 6.4) for extreme data with high N and low K uptake. The most frequent N:K ratio ranged from 1.8:1 to 2.4:1 (25% and 75% quartiles; Fig. 2.4B.1). The N:K distribution for ARG region was positively skewed with maximum N:K values close to 6.4:1, KS was more symmetric, and IN was more peaked with more concentrated N:K values from 1.5:1 to 2.0:1 units. Upper boundary for the N:K ratio was 6.25:1 units indicating a maximum K dilution in the plant relative to N; while the lower boundary was close to 1.25:1 depicting a maximum K accumulation. When residuals from the model were compared with $\%K_{\text{stover}}$ and $\%K_{\text{seed}}$, both were statistically significant ($p < 0.001$) but variations in $\%K_{\text{stover}}$ explained most of the variation of N:K relationship compared with $\%K_{\text{seed}}$ ($R^2 = 0.57$ and 0.32 , respectively; Fig. 2.4C).

DISCUSSION

Yield related traits: plant nitrogen uptake, NHI and $\%N_{\text{seed}}$

The broad variation documented for seed yield and biomass is a reflection of the large G x E x M interaction. Notwithstanding this large variability, the N efficiency term for the overall dataset was comparable to the one previously documented by several researchers, i.e. $0.0125 \text{ Mg seed yield kg}^{-1} \text{ N}$ (Salvagiotti et al., 2008; Rotundo et al., 2014; Collino et al., 2015). In a previous study, Sadras (2006) stated that variations in N efficiency (yield-to-N uptake) in legumes could be largely attributed to concomitant changes in $\%N_{\text{seed}}$ and NHI (with similar relative changes). In the present study, yield-to-uptake relation for N showed a significant

relation with NHI regardless of the G x E x M interaction. The latter result is supported by previous knowledge that NHI in legumes is more sensitive to changes in environmental conditions as related to other species (Chapman and Muchow, 1985).

For soybean, HI has been reported as a stable variable across different environments (Spaeth et al., 1984; Pedersen and Lauer, 2004). In other crops such as maize (Ciampitti and Vyn, 2012), wheat (Desai and Bhatia, 1978) and oat (*Avena sativa* L.; Rattunde and Frey, 1986), the interaction between N and dry matter allocation in seeds has been documented through the association between NHI and HI. In the abovementioned crops (i.e., cereals) the source of N is not totally guaranteed since it is dependent of the N soil availability (mineral- and fertilizer-N) in contrast with legumes where BNF is the main N source with considerable variation in the amount of N fixed (Collino et al., 2015). Hence, variations in the yield-to-plant N uptake relationship can be mainly accounted by changes in %N_{seed} as has been already reported (Sadras, 2006).

Genotype, environment and management effects

In a recent research study investigating G x E variations in N traits (Rotundo et al., 2014) for US and ARG soybean genotypes, plant N uptake was not influenced by the genotype factor regardless of the yield environment explored (from low – 2 Mg ha⁻¹ to high-yielding - 5 Mg ha⁻¹). For the same research study, changes in %N_{seed} depended on the dataset evaluated; ARG cluster portrayed a significant effect of this factor; while US cluster resulted primarily influenced by the genotype. Our dataset reflect the effect of combining diverse locations, portraying a large environmental effect on %N_{seed} and P-K within the same organ. Historical improvements in HI (Rowntree et al., 2014; Suhre et al., 2014; Jin et al., 2010; Morrison et al., 1999) and biomass

(Rowntree et al., 2014) were previously documented at varying maturity groups (MGs) but similar genetic yield gain rate was reported for both ARG and US from 1980 to 2015 (de Felipe et al., 2016). Larger genotypic variation effect on the HI and biomass for our database could be related to differences in MGs employed between locations. Nutrient partitioning indices followed a similar trend as depicted by the nutrient concentration in seed and stover fractions, largely influenced by the environment. For NHI, historical improvement has been associated with seed yield increases during the last several decades according to a retrospective study testing Chinese germplasm (Jin et al., 2011). For the US, a similar historical study, utilizing soybean varieties from the last 90 yrs was conducted by Long (2013) and documented improvements in NHI as yield increases (also associated to the newly released genotypes). For our database, environment and the G x E x M interaction and residuals accounted for a large proportion of the variation for several traits. Therefore, further research should explore the effect of diverse genotype on N traits.

Nutrient partitioning and harvest index

The trend in this relationship reflects the influence of the specific plant organ %N (%N_{seed}:N_{stover} ratio) on NHI. The latter implies the effect on the specific-organ %N, as related to the potential minimum and maximum attainable %N, could exert an influence in the N remobilization and N partitioning processes (as expressed by NHI). The outcome of this study support the concept presented by Lawn (1989) regarding dry mass and N partitioning (HI and NHI, respectively). The proportion of N partitioned to the seeds at maturity is correlated with the total amount of N in vegetative parts at R5 that will provide the main source of N for seeds (Zehier et al., 1982). As the concentration of N in vegetative parts increase, the developing seeds

obtain more of its N from remobilization and less from N uptake or BNF processes. Egli et al. (1983) arrived to similar conclusions but also testing different soybean genotypes in water stress conditions without significant changes in %N_{seed}. However, changes %N_{stover} was significantly different across environments affecting the proportion of N partitioned from vegetative parts.

In this study, the %N_{stover} presented a 7-fold greater variation relative to %N_{seed} (660% for %N_{stover} vs. 92% %N_{seed}). Therefore, %N_{seed} seems to be a more conservative trait, as opposed to the %N_{stover}, which indicates the ability of the plant to maintain seed N demand. In soybean, this functionality is mostly given by the leaves acting as N reservoir and to a less extent by the stem (Pierozan et al., 2015; Troedson et al., 1989). Meanwhile, in other crops, such as maize or sorghum, the stem functioning as an N reservoir and in the regulation of N translocation is more important than leaves when plants are growing at different N level conditions (van Oosterom et al., 2010; Ciampitti et al., 2013; Ciampitti and Prasad, 2016).

Following this rationale, attaining high HI is needed to increase N allocation and partitioning from the stover to the seeds. However, this approach is still unclear and will likely be the focus of future research because of the complexity behind the source-sink relationship in soybean. An option for improving N allocation to the seeds (increasing NHI) at comparable HI values is by increasing N remobilization from vegetative tissues as a soybean breeding goal. However, there are some physiological implications with N translocation from leaves related to leaf metabolites involved in the regulation of N partitioning during the reproductive stages (Franceschi and Giaquinta, 1983; Klauer et al., 1996).

The approach proposed by Lawn (1989) is an useful tool to investigate biomass and nutrient partitioning from a physiological standpoint. In the present study, the model predicted adequately %nutrient in seeds and stover fractions for N and K (Fig. 2.3A, C). On the opposite,

the PHI:HI model overpredicted the $\%P_{\text{seeds}}$ and $\%P_{\text{stover}}$, which could also be connected to the variation encountered on the data across all environments evaluated. As observed with NHI, the curvilinear relationship for PHI also indicates that more translocation of dry mass to the seeds is not proportional to P translocation to the seeds. Hence, it seems that soybeans are limited on the P partitioning from stover to seeds at high HI values. P supply to achieve target $\%P_{\text{seeds}}$ for the growing seeds would be provided by the stover through the regulation of $\%P_{\text{stover}}$ since $\%P_{\text{seeds}}$ resulted in a relative stable variable among the data sets. Even though in this data set the stover was not fractioned into leaves and stems, the former has been suggested as the primary source of remobilized P (Ao et al., 2014; Bender et al., 2015).

For K, the model prediction showed an adequate fit but the overall KHI:HI response presented more variation, may be related to the ample range documented in KHI (reported to range from 0.30 to 0.59 units; Hanway and Weber, 1971) and also to the late-season K dynamic in soybean plants. For instance, variations related to moments of leaf sampling may explain different K content at similar growth stage since losses related to leaching from vegetative organs of the standing plants are largely due to precipitation and leaf senescence (Schomberg and Steiner, 1999; Oltmans, 2012). In order to reduce this variability, quantification of K uptake at R6, when the crop has reached maximum nutrient content in standing biomass (Bender et al., 2015) might be an alternative. Also, K partitioning and accumulation in seeds represent a lower and stable percentage relative to the stover fraction. Thus, concentration in seed and stover tends to be more alike providing an explanation for the linearity of the KHI:HI relationship.

N:P and N:K stoichiometry

Changes in N:P and N:K ratios were associated with changes in plant P and K uptake, respectively. Similar results for N:P ratio were found by other authors when analyzing several field crops (Sadras, 2006; Ciampitti and Vyn, 2014). Additionally, P is closely related to N (Araújo and Teixeira, 2003) due P deposition (phytate) with protein bodies in the seeds (Bewley and Black, 1994). Phytate acts as an important source of phosphate and mineral elements, important for seed germination but also alters the bioavailability of certain dietary minerals in monogastric animals (Cheryan and Rackis, 1980). However, P dynamics and its interaction with N during seed filling period have not yet been fully investigated.

Variations in N:P and N:K ratios were closely tied to changes in the stover nutrient concentration. The primary factor behind changes in P and K stoichiometry was the differential partitioning between seeds and stover. Despite the similarity in nutrient ratios among regions, data distribution showed different extreme values that might be associated with imbalanced nutrient situations (e.g., high N:K ratios in ARG). This approach would be useful for detecting nutrient limitations (Koerselman and Meuleman, 1996; Salvagiotti et al., 2012).

CONCLUSIONS

Nitrogen harvest index was the main variable that explained the yield-to-uptake for N in soybean when studying seed yield generation using Equation 2.1. The relationship nutrient HI:HI relationship was well explained using the approach proposed by Sinclair (1998), not only for N but also for P in a similar fashion and for K with a different trend. Results emphasize the fact that efforts to increase seed yield and HI must be accompanied by improvement in processes associated with nutrient uptake, and remobilization/utilization taking into account the seed:stover

stoichiometry. Plant N uptake was strongly related to P and K uptake, and in both relationships, stover rather than seed concentration explained variations in these relationships. This implies that nutrient concentrations in the vegetative fraction can act either as storage or supply based on the status and target concentrations of P and K in the seed organ.

Further studies should pursue an in-depth characterization of the HI and nutrient HI response models and its relationship with the seeds and stover nutrient concentrations in order to characterize G x E x M variability for achieving high-yielding soybean farming systems, and evaluate the effects of this partitioning in nutrient cycling in agricultural systems.

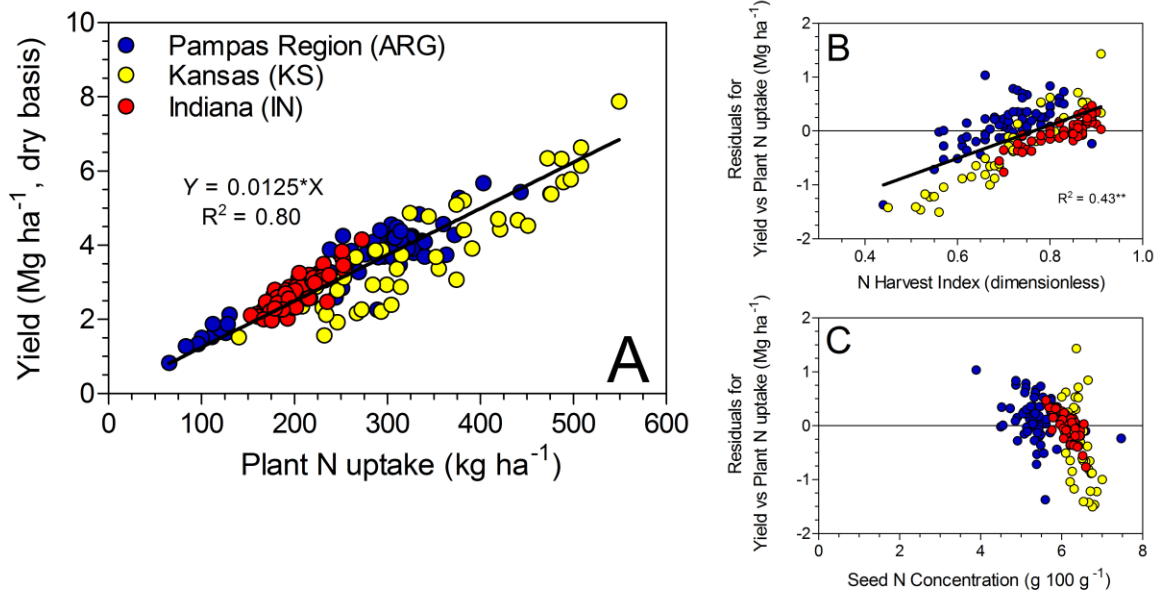


Figure 2.1: Relationship between seed yield and plant N uptake for pooled data (n = 167). The solid line represents the curve with y-int forced to zero for pooled data with a slope of 0.0125 Mg seed kg⁻¹ N (A). Relationship between residuals of the linear model fitted in (A) and N harvest index (B) and seeds N concentration (C) for each data set analyzed. Points with different colors are from ARG (blue; n = 68), KS (yellow; n = 45) and IN (red; n = 54).

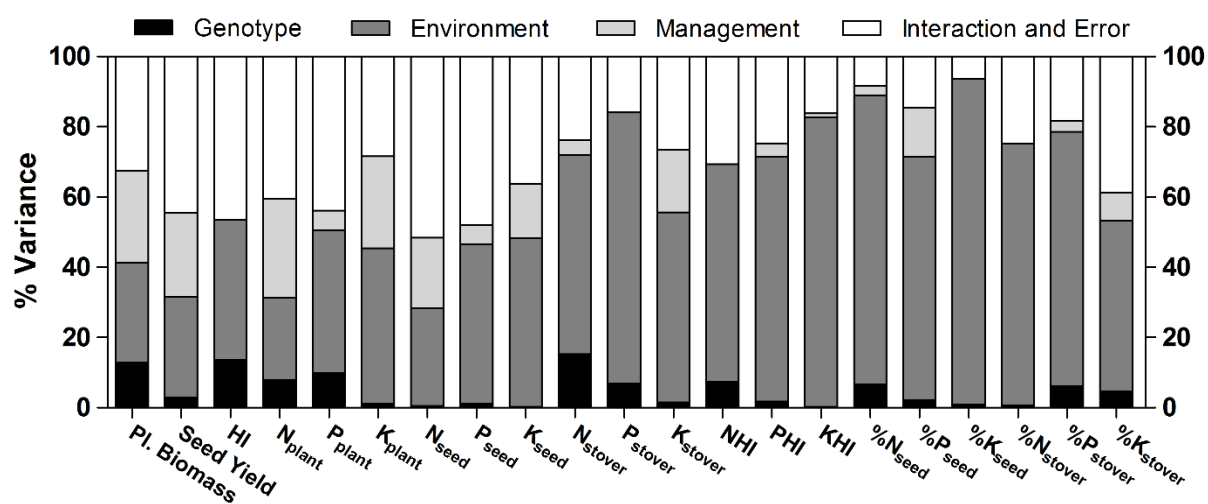


Figure 2.2: Variance partitioning for genotype, environment, management and the interaction with error of the total variance for each variable in the analysis.

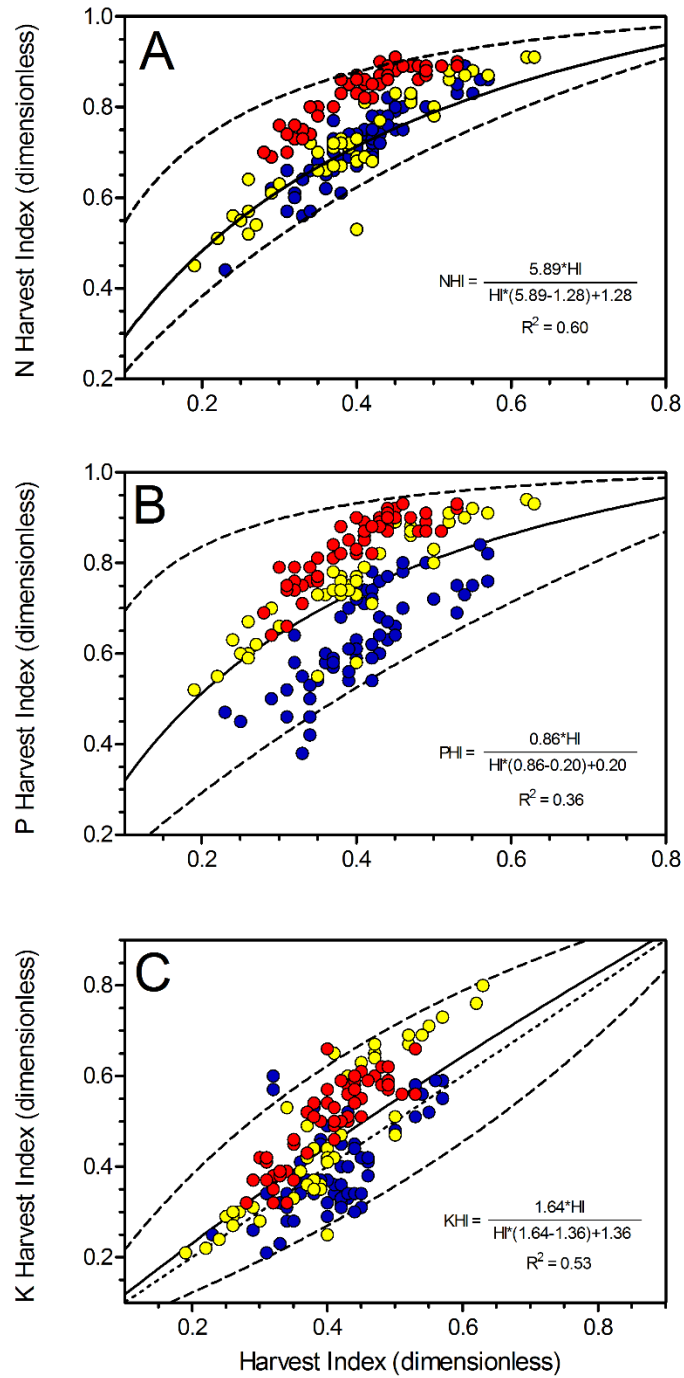


Figure 2.3: Relationship between N harvest index (NHI) (A), P harvest index (PHI) (B) and K harvest index (KHI) (C) and harvest index (HI) for the metadata (n = 167). Solid line represents the fitted model of Equation 1.4 and the dashed lines represent the 95% confidence intervals. Dotted line in Fig. 1.2C represents the 1:1 ratio when parameters of the Equation 1.4 are equals. Points with different colors are from ARG (blue; n = 68), KS (yellow; n = 45) and IN (red; n = 54). Bubble sizes represent different ranges of seed yield.

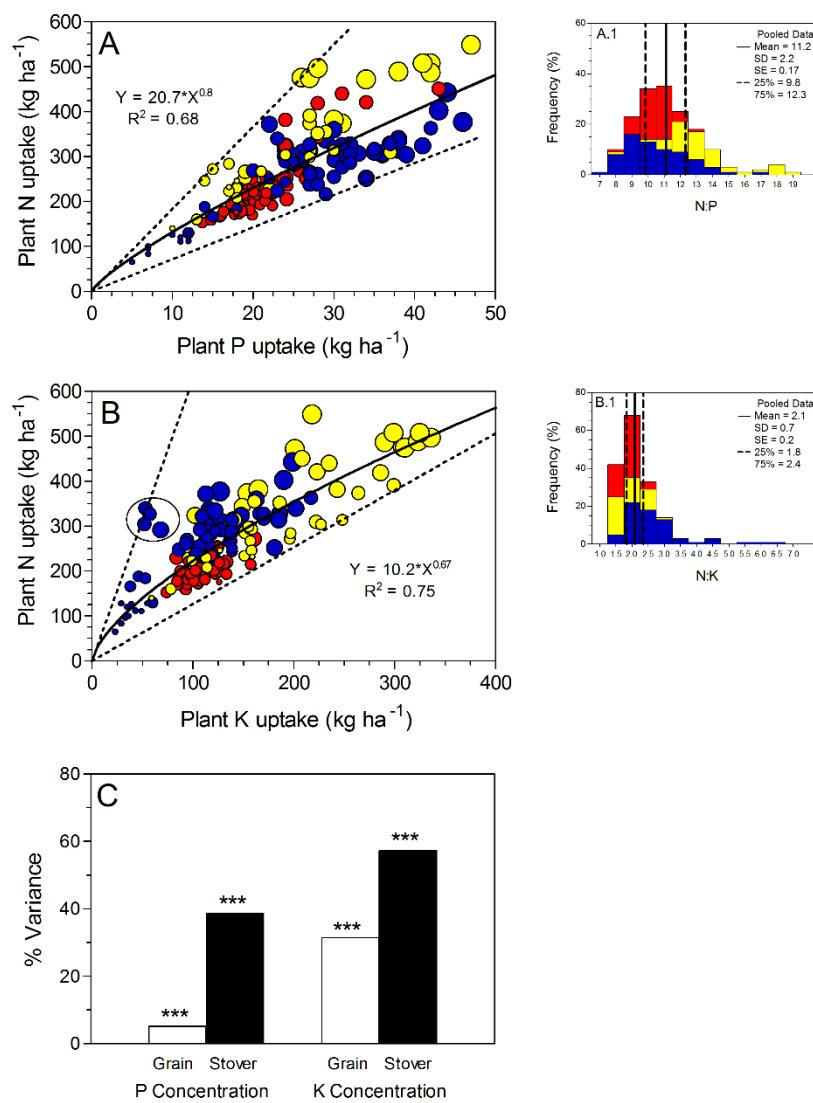


Figure 2.4: Plant N uptake as a function of plant P (A) and K uptake (B). Bubble sizes represent different ranges of seed yield. Frequency distribution of N:P (A.1) and N:K (B.1) ratios. Percentage of variance (R^2) provided by the linear regression between residuals of Fig. 1.3A as a function of seed and stover P concentration, and residuals of Fig. 1.3B as a function of seed and stover K concentration (C). n.s. is not significant; * $p < 0.001$. Outliers are encircled and were excluded from the fitted dataset.**

Table 2.1: Locations, years, soil characteristics, water supply, plant density, genotypes, and main characteristics for each data set.

| Data set | Location | | Year | Soil | | | | | | Water supply | Plant density | Genotype | | | Main characteristics | |
|--------------------------|------------------|------------------|-----------|------------|------|-------------------|--------------------------------|------|-------|------------------------|--------------------|----------|------------------------------------|------------|--|--|
| | | | | Soil type | pH | OM ¹ | NO ₃ ⁻ N | P | K | | | Number | Release year | MG | | |
| | | | | | | g g ⁻¹ | ppm | ppm | | | pl m ⁻² | | | | | |
| Pampas Region, Argentina | Oliveros | 32°34'S; 60°51'W | 2009/10 | Silt loam | 5.7 | 2.1 | 26.7 | 26.0 | - | Rainfed/ Irrigation | 30 (16 – 53) | 6 | 2005-2009 | III, IV, V | Soybean crops testing under rainfed conditions without nutrient limitations across several fields. | |
| | | | 2009/10 | Silt loam | 5.0 | 2.1 | 27.0 | 9.0 | - | | | | | | | |
| | | | 2010/11 | Silt loam | 6.0 | 2.6 | 37.7 | 42.0 | - | | | | | | | |
| | | | 2011/12 | Silt loam | 5.9 | 2.2 | 19.7 | 24.0 | - | | | | | | | |
| | | | 2012/13 | Silt loam | 5.5 | 2.7 | 40.0 | 26.0 | - | | | | | | | |
| | Sgto Cabral | 33°25'S; 60°37'W | 2009/10 | Silt loam | 5.8 | 2.3 | 12.9 | 19.0 | - | | | | | | | |
| | Serodino | 32°36'S; 60°57'W | 2009/10 | Silt loam | 5.6 | 2.5 | 19.0 | 18.0 | - | | | | | | | |
| | Aldao | 32°42'S; 60°49'W | 2010/11 | Silt loam | 6.2 | 2.4 | 4.3 | 5.5 | - | | | | | | | |
| Carcaraña | 32°51'S; 61°09'W | 2010/11 | Silt loam | 6.0 | 2.5 | 46.1 | 11.7 | - | | | | | | | | |
| Kansas, US | Scandia | 39°49'N 97°50'W | 2014 | Silt loam | 6.3 | 3.1 | 22.5 | 18.2 | - | Rainfed | 30 (16 – 49) | 1 | 2008 | IV | Row spacing, inoculation, plant density, fertilization strategy | |
| | Hutchinson | 37°55'N 98° 1'W | | Silt loam | 4.9 | 2.7 | 12.9 | 21.8 | - | Irrigated | | | | | | |
| | | | | Loam | 5 | 1.5 | - | 62.5 | 156.7 | Rainfed | | | | | | |
| Indiana, US | West Lafayette | 40°25'N; 86°55'W | 2011-2012 | Fine-silty | 6.48 | 2.9 | - | 43 | 175 | Rainfed | 38 (12 – 60) | 9 | 2002, 2004, 2005, 2007, 2008, 2011 | II, III | Varieties, biomass and nutrient uptake and partitioning | |

¹ Organic matter

Table 2.2 Descriptive statistics (minimum, mean, maximum, 25 & 75% percentile, and standard deviation – SD) of total plant biomass (dry basis), seed yield (dry basis), harvest index (HI), N,P and K nutrient harvest index, seed nutrient concentration and uptake in total plant, seed and stover fractions.

| Variable | Unit | Minimum | 25% Percentile | Mean | 75% Percentile | Maximum | SD |
|----------------------|-----------------------|---------|-------------------|------|-------------------|---------|------|
| Plant biomass | Mg ha ⁻¹ | 2.0 | 6.6 | 8.6 | 10.8 | 15.5 | 2.9 |
| Seed Yield | Mg ha ⁻¹ | 0.8 | 2.5 | 3.4 | 4.1 | 7.9 | 1.2 |
| HI | dimensionless | 0.19 | 0.35 | 0.40 | 0.45 | 0.63 | 0.08 |
| N _{plant} | kg ha ⁻¹ | 65 | 202 | 267 | 315 | 549 | 93 |
| P _{plant} | kg ha ⁻¹ | 5 | 18 | 24 | 30 | 47 | 8 |
| K _{plant} | kg ha ⁻¹ | 23 | 99 | 133 | 157 | 336 | 62 |
| N _{seed} | kg ha ⁻¹ | 45 | 154 | 199 | 226 | 501 | 69 |
| P _{seed} | kg ha ⁻¹ | 3.3 | 14 | 17 | 20 | 44 | 5.8 |
| K _{seed} | kg ha ⁻¹ | 7.9 | 40 | 57 | 66 | 165 | 27 |
| N _{stover} | kg ha ⁻¹ | 16 | 32 | 69 | 103 | 166 | 42 |
| P _{stover} | kg ha ⁻¹ | 1.3 | 2.7 | 6.9 | 10 | 23 | 5.0 |
| K _{stover} | kg ha ⁻¹ | 13 | 44 | 76 | 96 | 218 | 45 |
| NHI | dimensionless | 0.44 | 0.69 | 0.75 | 0.85 | 0.91 | 0.10 |
| PHI | dimensionless | 0.38 | 0.63 | 0.73 | 0.86 | 0.94 | 0.13 |
| KHI | dimensionless | 0.21 | 0.34 | 0.45 | 0.56 | 0.80 | 0.13 |
| %N _{seed} | g 100 g ⁻¹ | 3.89 | 5.45 | 5.92 | 6.34 | 7.47 | 0.58 |
| %P _{seed} | g 100 g ⁻¹ | 0.32 | 0.46 | 0.53 | 0.59 | 0.70 | 0.08 |
| %K _{seed} | g 100 g ⁻¹ | 0.64 | 1.30 | 1.70 | 2.09 | 2.48 | 0.48 |
| %N _{stover} | g 100 g ⁻¹ | 0.53 | 0.93 | 1.27 | 1.51 | 3.81 | 0.45 |
| %P _{stover} | g 100 g ⁻¹ | 0.04 | 0.07 | 0.13 | 0.18 | 0.26 | 0.06 |
| %K _{stover} | g 100 g ⁻¹ | 0.27 | 1.14 | 1.42 | 1.62 | 4.36 | 0.46 |

Chapter 3 - Interplay between nitrogen fertilizer and biological nitrogen fixation in soybean: implications on seed yield and biomass allocation

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ABSTRACT

Legumes rely on soil mineral nitrogen (N) and biological N fixation (BNF). The interplay between these two sources is biologically interesting and agronomically relevant as the crop can accommodate the cost of BNF by five non-mutually exclusive mechanisms, whereby BNF: reduces shoot growth and seed yield, or maintains shoot growth and seed yield by enhanced photosynthesis, or reduced root:shoot ratio, or maintains shoot growth but reduces seed yield by reducing the fraction of shoot biomass allocated to seed (harvest index), or reducing concentration of oil and protein in seed. We explore the impact of N application on the seasonal dynamics of BNF, and its consequences for seed yield with emphasis on growth and shoot allocation mechanisms. Trials were established in 23 locations across the US Midwest under four N conditions. Fertilizer reduced the peak of BNF up to 16% in applications at the full flowering stage. Seed yield declined 13 kg ha⁻¹ per % increase in BNF. Harvest index accounted for the decline in seed yield with increasing BNF. This indicates the cost of BNF was met by a relative change in dry matter allocation against the energetically rich seed, and in favor of energetically cheaper vegetative tissue.

INTRODUCTION

Globally, soybean [*Glycine max* (L.) Merr.] is a major source of protein and oil. In the US, soybean is grown in a range of latitudes and environments representing 29% of the national crop acreage (USDA, 2017). Significant breeding effort during the last century sought to improve seed yield and maintain seed protein (Jin et al., 2010). One of the challenges to further improve soybean seed yield is the high demand of nitrogen (N) in comparison to cereals and oilseed crops (Balboa et al., 2018; Tamagno et al., 2017). Legumes rely on two sources of N: mineral N from soil and biological fixation (BNF); the proportion from each source varies with environmental and soil conditions including temperature (Trang and Giddens, 1980), soil moisture (de Silva et al., 1996; Sadras et al., 2016), soil pH (Ferguson et al., 2013), mineral soil N (Eaglesham et al., 1982), and genotype of both bacteria (Danso et al., 1986) and crop (Purcell et al., 1997; Sadras et al., 2016). In soybean, BNF is greater in genotypes with longer reproductive periods reflected in maturity group (MG) (Patterson and LaRue, 1983).

Biological N fixation requires plant's reduced carbon (C) and energy, as reviewed by Kaschuk et al. (2009). For soybean, BNF requires 6-7 g C g⁻¹ N in comparison to 4 g C g⁻¹ N for assimilation of mineral N; integrated over the growing season the difference in cost is substantial, with potential implications for seed yield and seed protein or oil concentrations. The cost of BNF can be partially compensated by increase in photosynthesis of plants associated with rhizobia¹³ or shifts in allocation of biomass. For instance, nodulated roots accumulated less biomass compared with plants growing with high soil N supply (Salon et al., 2001) and lower biomass partitioning to seeds associated with increasing BNF (Sadras et al., 2016). Thus, the crop can accommodate the cost of BNF by five non-mutually exclusive mechanisms, whereby N fixation: (a) reduces shoot growth and seed yield, or maintains shoot growth and seed yield by

(b) enhanced photosynthesis (Kaschuk et al., 2009), or (c) reduced root:shoot ratio (Lambers, 1983), or maintains shoot growth but reduces seed yield by (d) reducing seed oil and protein concentration in seed, or (e) the fraction of shoot biomass allocated to seed (i.e., harvest index; HI).

Further, there is an agronomic interest on the role of mineral N to support high seed yield (Cafaro La Menza et al., 2017; Tamagno and Ciampitti, 2017) and avoid protein dilution (Medic et al., 2014; Rotundo et al., 2016). A recent review of Mourtzinis et al. (2018) concluded that N fertilization has a small and inconsistent effect on soybean seed yield. This conclusion is, however, largely based on generic trials where coarse fertilization regimes were established to shift the contribution of mineral N and BNF. In contrast, a full-N treatment devised with a careful experimental protocol to ensure an ample N supply during the entire crop season increased soybean seed yield by 11% in relation to unfertilized controls, with a range from no effect for stressful environments (ca. 2500 kg ha⁻¹) but increases of 900 kg ha⁻¹ increase in high potential environments (ca. 6000 kg ha⁻¹; Cafaro La Menza et al., 2017).

The goal of this study was to investigate the effect of fertilizer N application on BNF and its implications for soybean seed yield and seed protein concentration. We tested the hypothesis that the cost of N fixation is mediated by reduced biomass, reduced allocation to seed captured in the HI (Hay, 1995), and reduced concentration of protein and oil in seed. Quantification of these effects will provide insights of BNF impact on crop C and N economy, and will contribute to explain the apparent inconsistency in soybean seed yield responses to N fertilization.

MATERIALS AND METHODS

Experimental sites and treatments

During the 2016 growing season, soybean N fertilization studies were replicated in 23 sites across the US Midwest in a latitude range from 34°16' N to 48°14' N and from 90°29' W 98°53' W (Fig. 3.1, Table 3.1). Due to the range of latitudes between locations, the length of the growing season differs among sites. Thus, sowing dates and MG were considered following local management practices and recommendations which ranged from 0 to IV due to the large range of latitude in the locations (Mourtzinis and Conley, 2017). The seeding rate of 300,000 seeds per hectare, targeted maximum seed yield. Crops were rainfed and received supplementary irrigation to avoid severe water stress (Table 3.1). On-site weather stations recorded daily temperature, precipitation, and relative humidity; the vapor pressure deficit (VPD, kPa) was estimated using the maximum daily temperature and relative humidity (Howell and Dusek, 1996). Soil parameters from every location are presented in Table 3.1. Data for percentages of clay and sand, organic matter (OM), soil pH and cation exchange capacity (CEC) was extracted from the California Soil Resource Lab (<http://casoilresource.lawr.ucdavis.edu>, accessed 11 June 2018) using latitude and longitude coordinates from each experiment.

Four treatments were established: an unfertilized control, and 112 kg N ha⁻¹ as urea (46-0-0 N-P-K,) at one of three stages: at sowing, at V4 (fourth-leaf), and between R2 (full flowering) to R3 (beginning of pod formation; Fehr and Caviness, 1977). The experimental design at each location was a randomized complete block with three replicates. Plot size was 8.4 m long by eight rows at 0.76 m row spacing. The supply of other nutrients was done with N-free fertilizer.

Phenology, biomass, seed yield, harvest index, seed protein and oil concentration

Plant development stages in relation with calendar time is usually referred to as phenology. Phenological stages were recorded during the season following Fehr and Caviness (1977). Shoot biomass samples were collected at the R8 stage (full maturity) from 1.6 linear m and fractioned into stem, leaves, and seeds. The relative proportion of seeds to the total shoot biomass was quantified as the harvest index (HI; Donald and Hamblin, 1976). Variations on this ratio can be associated with the influence of the environmental effects on seed yield and biomass production. Seed yield was collected from two-central rows at maturity and adjusted to 13.5 g 100 g⁻¹ seed moisture content. Seed samples were collected from harvest for oil and protein determination by near infrared (NIR) spectroscopy using a completely automated Fourier Transform-IR imaging Microscope (Hyperion 3000, Bruker Optics, Ettlingen, Germany) and a sample of >50 seeds. Seed protein and oil concentrations are reported on dry weight basis (Table 3.2).

Seasonal dynamics of BNF

Biological N fixation was measured four times, at V4 (fourth leaf), R2 (full flowering)-R3 (beginning of pod formation), R6 (full seed), and R8 (full maturity) using main stem samples. Stems were dried at 65° C until constant weight and ground to pass through a 2-mm sieve. The BNF percentage was calculated as the relative abundance of ureide-N (RAU) in the main stems using the procedure of Hungria and Araujo (1994). The RAU was calculated as a function of ureides nitrate-N (NO₃-N) concentration (Schweiger et al., 2012).

$$\text{RAU (\%)} = \frac{4 \times \text{Ureide concentration}}{[(4 \times \text{Ureide concentration}) + \text{NO}_3^- \text{ concentration}]} \times 100$$

Equation 3.1

Time units used to measure the progress of RAU during the season was thermal units (degree-days; °Cd) to account for thermal differences in growing conditions and be independent from the temperature in which different developmental stages occurs. A degree-day is the result from every degree on the daily mean temperature above the base temperature (Monteith, 1977). Thus, cumulative thermal time was calculated as:

$$\text{Thermal Time (°Cd)} = \sum \left(\frac{T_{\max} + T_{\min}}{2} - T_{\text{base}} \right) \quad \text{Equation 3.2}$$

where T_{\max} and T_{\min} is the maximum and minimum daily air temperature (°C), and T_{base} is the base temperature (8°C; Wu et al., 2015).

Changes in RAU during the growing season has been described as a sigmoidal pattern with a slow increase early in the season and a maximum attainable between R5 (beginning of seed filling) and R6 (full seed) (Herridge, 1982). However, owing to the large variation in genotypes and growing conditions, RAU at R6 varied widely. To account for this variation, we used RAU at R6 in unfertilized controls to split data into three groups: below the 25th quartile (low BNF), between the 25th-75th quartiles (medium BNF), and above the 75th quartile (high BNF). The low BNF comprised five sites with RAU below 72%; the medium BNF included twelve sites with RAU from 72 to 88%, and the high BNF group comprised of six sites with RAU above 88%. For the data combined for each group, the seasonal RAU evolution was described with the beta growth function (Yin et al., 2003) with three parameters:

$$\text{RAU (\%)} = \text{RAU}_{\max} \left(1 + \frac{t_{\max} - t}{t_{\max} - t_m} \right) \left(\frac{t}{t_m} \right)^{\frac{t_{\max}}{t_{\max} - t_m}} \quad \text{Equation 3.3}$$

where t is the thermal time from V2 (vegetative leaf), RAU_{max} is the maximum RAU at thermal time t_{max} , and t_m is the thermal time for maximum RAU growth rate. Biological meaning on parameters allowed us to make inferences on the magnitude of the N treatments on the RAU dynamics by statistical comparisons. Differences between parameters of Equation 3.2 were tested using the Akaike's Information Criteria (AIC) by performing pairwise comparisons of individual curves against a global fit. Maximum rate, $t_{0.5}$, and the AUC were compared using the 95% confidence interval. Both RAU and thermal time were estimated through least squares mean analysis by fitting a mixed model with PROC MIXED procedure (SAS Institute, 2012) (lsmeans statement) to adjust corrected means to the factors of the model. For this analysis, treatment and locations were considered as fixed factors, and block was nested within location as a random factor. The goodness of fit of the model was assessed with the coefficient of determination (R^2) and the standard deviation of residuals (Syx) (Archontoulis and Miguez, 2015).

Using Equation 3.3, we derived three related traits: the AUC to integrate seasonal N fixation (Gagnon and Peterson, 1998) normalized to the maximum of the data set; $t_{0.5}$ the thermal time when RAU is 50% of RAU_{max} ; and the maximum rate of RAU expressed in changes of % units of RAU per unit of thermal time ($^{\circ}Cd$; Yin et al., 2003):

$$\text{Maximum Rate } (\% \text{ Cd}^{-1}) = \frac{2t_{max}-t_m}{t_{max}(t_{max}-t_m)} \left(\frac{t_m}{t_{max}} \right)^{\frac{t_m}{t_{max}-t_m}} RAU_{max} \quad \text{Equation 3.4}$$

The first derivative of Equation 3.3 with respect to thermal time can be solved to calculate the RAU rate changes across the growing season (Yin et al., 2003):

$$\text{RAU Rate (\% Cd}^{-1}\text{)} = \text{Max. Rate} \left(\frac{t_{\text{max}} - t}{t_{\text{max}} - t_m} \right) \left(\frac{t}{t_m} \right)^{\frac{t_m}{t_{\text{max}} - t_m}} \quad \text{Equation 3.5}$$

Same approach has been utilized to describe other biological process such as N uptake rate in corn (Osterholz et al., 2017) or grain growth rate (Meade et al., 2013).

Analysis of treatment effects and associations between traits

Analysis of variance (ANOVA) was used to investigate effects of treatments on crop traits (seed yield, biomass, HI, RAU_{R6}, seed protein and oil concentration). Sources of variation in ANOVA included N treatment, location, and their interaction as fixed factors, and block as a random effect nested within location; this analysis was implemented by using the R software (version 3.4.0, lme4 package, lmer function)(Bates et al., 2015; R Core Team, 2018).

The effects of BNF on seed yield, biomass, HI, seed protein and oil concentration were analyzed in two steps. First, due to the geographical distribution of the experiments, responses on crop traits are confounded with the different duration of the developmental stages. Thus, effects of phenology were captured with non-linear models:

$$Y = aX + b \text{ when } X < TT_o \quad \text{Equation 3.6a}$$

$$Y = dX \text{ when } X \geq TT_o \quad \text{Equation 3.7b}$$

where Y is the trait, X is the thermal time to R6 (°Cd), and a, b, TT_o, and d, are parameters. Next, linear regressions and quantile regressions were fitted between residuals of these models and RAU_{R6}. This simple approach on the use of residuals allows to netting out (Greene, 2003) the effect of the phenology on the traits observed when are regressed against RAU_{R6}. There are

relationships on other parts of the distribution of the response variable that can provide more complete view of the processes studied besides of the mean effect observed. Slopes from quantile regression analysis estimate the changes at the maximum and minimum response that can be missed when other regression methods are used (Cade and Noon, 2003). Thus, regressions for 0.99 and 0.01 quantiles capture the boundaries of the relationships, and were fit in R (quantreg package; Koenker, 2017). The rest of linear and non-linear regression analyses, computation of AUC, and estimation and comparison of parameters from Equation 3.3 and 3.5 were performed using GraphPad Prism (Motulsky and Christopoulos, 2003).

Principal component analysis (PCA) was used to analyze general associations between traits allowing the identification of any grouping association within the data set when environmental and crop attributes are analyzed together (Ju et al., 2017). Data were classified according to MG, which in turn had a geographical correlation (Table 2.1). Traits included RAU_{max} , t_{max} , maximum rate of RAU evolution, seed protein and oil concentration, AUC, residuals from seed yield, biomass, and HI vs thermal time to R6 relationship, and soil attributes (clay, sand, organic matter, pH, and CEC). Principal component analysis was fit using the “FactoMineR” package in R (Lê et al., 2008). Pearson correlation coefficients were calculated to complement associations found in the PCA.

RESULTS

Effect of N fertilizer on N fixation

Data for this study were collected from 23 different locations across the US Midwest during the 2016 growing season (Table 3.1; Fig. 3.1). Table 3.2 shows the values for the relative abundance of ureides in R6 (RAU_{R6}) for each location and treatment, Fig. 3.2

illustrates the seasonal dynamics of the relative abundance of ureides (RAU) for crops grouped in high, medium and low BNF, and Table 3.3 summarizes the parameters of the curves. The fitted model (Equation 3.2) returned R^2 between 0.62 and 0.87, with $P < 0.001$ in all cases.

Values collected for RAU_{R6} ranged between 42 to 93% (Table 3.2) and responded to all three sources of variation: treatment, location and their interaction ($P < 0.001$). Fertilizer reduced RAU_{max} in all BNF groups, but not where BNF was already low in the control treatment (Table 3.3). In the high BNF group, RAU_{max} dropped from 90% in controls to 75% in their fertilized counterparts (averaging all N treatments; Table 3.3); the reduction in BNF was larger when the application of N was delayed from vegetative to reproductive stages. In the medium group, RAU_{max} declined from 84% in controls to 68 in the V4 application and 74% in both sowing and R2 applications, with no clear effect of N application timing.

The reduction in RAU_{max} can be a consequence of a shorter time to peak RAU, a reduced rate or a combination of both effects. Combination of both traits contributed to reduced N fixation in the medium BNF group, as peak RAU and maximum rate were attained earlier in N-fertilized crops. In the low BNF group, N fertilizer reduced RAU rate but time of peak RAU was not affected. Reduction of the area under the curve (AUC) and the time when RAU reached 50% was observed from high to low BNF groups, however AUC was not affected by treatments (Table 3.3).

Fertilization treatments affected the dynamics of RAU rate. Both the timing of peak rate (t_m), and the timing when rate became negative (t_{max}) were delayed from low to high BNF groups (Fig. 3.2D-F). For the high and medium groups, reproductive N treatment was the most effective reducing both t_m and t_{max} hence contributing to an overall reduction of RAU_{max} .

Effect of N fixation on seed yield, biomass, harvest index, protein and oil concentration

Seed yield ranged from 3151 to 7175 kg ha⁻¹, seed protein concentration from 31.9 to 41.8 g 100 g⁻¹, and seed oil concentration from 16.7 to 23.9 g 100 g⁻¹ (Table 3.2). For these traits, ANOVA showed location was a significant source of variation ($P < 0.001$), with no effect of treatment and its interaction with location ($P > 0.05$). Total biomass ranged from 6093 to 11376 kg ha⁻¹ showing differences only among locations ($P < 0.001$). Harvest index ranged from 0.37 to 0.56, and was affected by both treatment ($P < 0.05$) and location ($P < 0.001$).

In this experiment, MGs were allocated to locations for agronomic relevance (Table 3.1). The dominant effect of location on crop traits is therefore confounded with crop phenology. For instance, the range of thermal time to R6 was 909 to 1733 °Cd. We thus fitted bilinear models to account for the effect of phenology on crop traits (Fig. 3.3A, C, D), and regressed the residuals against RAU_{R6} (Fig. 3.3B, D, F). Analysis of residuals showed seed yield declined at 13 kg ha⁻¹ per % of RAU_{R6} for the whole data set and 10 kg ha⁻¹ per % of RAU_{R6} ($P < 0.05$) for top yielding crops (0.99 quartile). Under stressful conditions leading to low HI (0.01 quartile), the rate of decline in HI with RAU_{R6} was 2.5 times larger than for the pooled data set. Oil seed concentration was negatively associated with RAU_{R6} ($P < 0.05$) and crops with higher oil concentration (0.99 quartile) were more responsive to RAU_{R6}. After removing the effects of phenology, biomass (Fig. 3.3F) and seed protein concentration (not shown) did not relate to RAU_{R6} (both $P > 0.05$).

The association between BNF traits, soil attributes, seed yield, biomass, HI, seed protein and oil concentrations, adjusted by the effects of phenology, were explored using PCA (Fig. 3.4) and estimating the Pearson correlation coefficients for their associations (Table A.1). Data

clustered with MG, reinforcing the dominant influence of phenology previously observed (Fig 3.3A, C, E). The MGs, from the shortest to the longest, grouped in the biplot along the first principal component.

Soil attributes were positively correlated in the first principal component, discriminating the locations with shorter maturity groups and higher values in soil attributes. Interestingly, AUC was negatively correlated with OM, pH, clay and sand percentage and the RAU_{R6} and rate were positively correlated with the OM and sand (Table A.1). Seed yield correlated positively with both HI and biomass and negatively with RAU_{max} , RAU_{R6} , and protein concentration. The AUC correlated positively with RAU_{max} and t_{max} and negatively with the maximum RAU rate.

DISCUSSION

Biological N fixation in soybean has been quantified at different scales, from field to country (Collino et al., 2015; Herridge et al., 2008; Hungria et al., 2005). The range of RAU in unfertilized controls in our study ranged from 48 to 93%. This compares with an average of 60% of N derived from BNF for the US (Herridge et al., 2008). In Argentina, BNF in 86 location-years averaged 60% and ranged from 12 to 90% (Collino et al., 2015). In Brazil, measurements in 6 environments returned an average of 81% and a range from 69 to 94% (Hungria et al., 2005). All these studies reflected a similar BNF ceiling around 90%, comparable to the maximum recently reported by Ciampitti and Salvagiotti (Ciampitti and Salvagiotti, 2018).

Nitrogen fertilization reduced BNF and increased seed yield by enhancing C allocation to seed

Phenology was the main source of variation in seed yield and traits associated with BNF clustered with maturity group (Fig. 3.2, 3.4). Soybean maturity group influences not only phenology but also growth, and allocation of biomass and nitrogen (Santachiara et al., 2017a). Variation in BNF with MG relates to both the duration of the reproductive period when BNF and biomass growth rate peak, and the delay in the exponential phase of BNF (Havelka et al., 1982; Keyser and Li, 1992; Patterson and LaRue, 1983). In our study, application of N reduced peak and altered dynamics of RAU during the season (Fig. 3.2), but phenology masked the association between N fixation and seed yield. After removing the dominant effect of phenology, crop yield declined with increasing BNF (Figs. 3.3, 3.4). This effect of maturity group has not been considered in previous comprehensive studies (Mourtzinis et al., 2018; Salvagiotti et al., 2008).

Reduced HI was the primary driver of the reduction in seed yield with increasing BNF, with an additional weak but significant reduction in seed oil concentration. Low yielding environments showed a steeper decline of HI with increasing BNF (Fig. 3.3D), highlighting the interaction with overall environmental conditions affecting dry matter allocation; stress during reproduction often reduces HI (Shiraiwa et al., 2006; Sinclair, 1998).

Changes in plant C allocation in association with BNF have been reported at different levels of organization and time scales. Reduction in BNF associated with phosphorus deficiency altered short-term allocation of C in lupin (*Lupinus luteus*), reducing photosynthesis:respiration ratio, and increasing the ratio between growth respiration and maintenance respiration (Kleinert et al., 2014). Likewise, low magnesium supply altered carbohydrate allocation in soybean, increasing sucrose and starch allocation to leaves that later limited nodule growth (Peng et al.,

2018). Decreases in biomass allocation in seeds for chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.) were reported with increasing BNF (Sadras et al., 2016; Voisin et al., 2002). Re-analyzing the data of Sadras et al. (2016), where 20 chickpea varieties were grown in 8 environments, HI declined with BNF at a rate of 0.0022 units per %BNF (Fig. A.1), in comparison with 0.0011 units per %BNF for soybeans in our study; similar to our trial, the decline in HI with BNF was larger for stressed chickpea crops.

Generically, plants require 1 g of glucose to produce either 0.33 g of lipid, 0.40 g of protein, or 0.83 g of carbohydrates (De Vries et al., 1974). Reducing oil concentration in seed is therefore an energetically effective way to meet the cost of BNF, as found in this study (Fig. 3.3E). This is in contrast to previous studies where seed protein concentration was reduced and oil concentration did not change in response to BNF (Purcell et al., 2004; Ray et al., 2006).

Our findings are in contrast to other studies where N fertilizer reduced BNF but did not increase soybean seed yield (Kaschuk et al., 2016; Santachiara et al., 2018). For example, Santachiara et al. (2018) found no seed yield response in heavily fertilized crops (600 kg N ha⁻¹ spread over the season) that reduced BNF to 16% in comparison to 69% in unfertilized controls. However, Santachiara et al. (2018) neither report seed yield in equivalent glucose nor changes in protein and oil concentrations. Results from these experiments can be influenced from soil variables influencing BNF activity. In our study, long-term stable soil attributes were included in the analysis. Organic matter was positively correlated with RAU_{R6} and the maximum RAU rate but negatively with the AUC (Table A.1). The negative association between AUC and soil organic matter might be attributed to the soil N mineralized from N organic fraction during the season (Thies et al., 1991). Collino et al. (2015) compared average BNF in soybean production

systems of Argentina and Brazil, and attributed the lower BNF in the former to better soil fertility.

Of the five putative mechanisms to account for the metabolic cost of BNF, enhanced sink-driven photosynthesis (Kaschuk et al., 2009) and reduced root:shoot ratio are the remaining hypotheses to explain the lack of seed yield response with reduced BNF in Santachiara et al. (2018). Implicit in the photosynthesis hypothesis is that soybean seed yield is sink-limited; although unlikely, it requires further research. Enhancing photosynthesis by increasing atmospheric carbon dioxide, Ryle et al. (1988) reported increases in nodule activity for shadowed white clover (*Trifolium repens* L.) plants but not in their non-stressed counterparts. It is likely that effects of photosynthetic rates on nodule activity depend on reserve carbohydrates (Walsh et al., 1998), suggesting a link with the differential trends in HI between favorable and poor environments observed in this study. An alternative, less explored mechanism for the maintenance of seed yield in crops relying on N fixation relative to fertilized crops is the reduction in root:shoot ratio; reduced root:shoot ratio is a generic response of plants to high availability of soil N (Lambers, 1983).

Agronomic and breeding implications

Soybean plays a relevant role in crop rotations (Sisworo et al., 1990) and is a major source of oil and protein worldwide. Improving BNF can be achieved by breeding and selection targeting the plant, the N-fixing bacteria, and better matching plant and bacteria (Denison, 2015; Emerich and Krishnan, 2009; Kiers et al., 2013). Selection for maintenance of BNF in dry soil has been proposed to improve seed yield of soybean under drought (Patterson and Hudak, 1996; Sinclair et al., 2007). Sinclair (2011) and Sinclair et al. (2007) combined ureide concentration in

petioles and acetylene reduction activity to test this proposition. Selected lines were compared with high-yielding commercial cultivars under broad environmental conditions. Two lines were identified that outperformed commercial checks under water deficit, but trade-offs were apparent under high yielding conditions. In this context, the trade-off between BNF and seed yield mediated by HI needs attention. Solving this trade-off needs quantification of the costs (seed yield reduction), agronomic and environmental benefit of BNF. Selection for high biomass partitioning to seed in genotypes growing under low concentration of soil nitrate represents a possible breeding strategy as higher rates of BNF are expressed. In both soybean (Santachiara et al., 2017b) and common bean (*Phaseolus vulgaris* L.; Hardarson, 1993), sensitivity of N fixation to soil mineral N varies with genotype. In alfalfa (*Medicago sativa* L.), selection for BNF improved plant growth (Barnes et al., 1988).

CONCLUSION

Seasonal characterizations over a wide range of agronomic and environmental conditions revealed that N application reduced maximum BNF at R6, particularly for late applications. At the crop level, soybean met the cost of BNF by a reduction in seed yield mediated by lower HI, particularly in stressful environments, and a secondary contribution of reduced seed oil concentration. The lower-level mechanisms underlying shifts in HI associated with BNF warrant further attention.

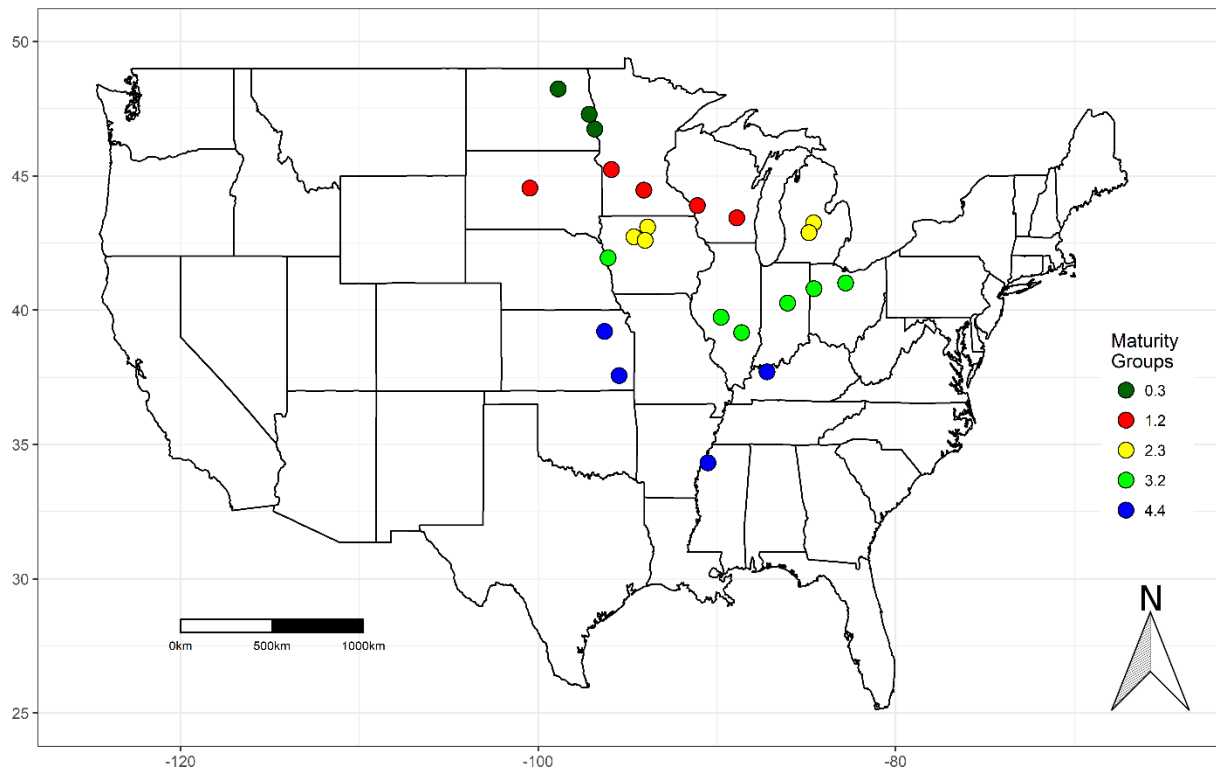


Figure 3.1: Map of the United States referencing all the experimental locations. Colors represent different maturity groups.

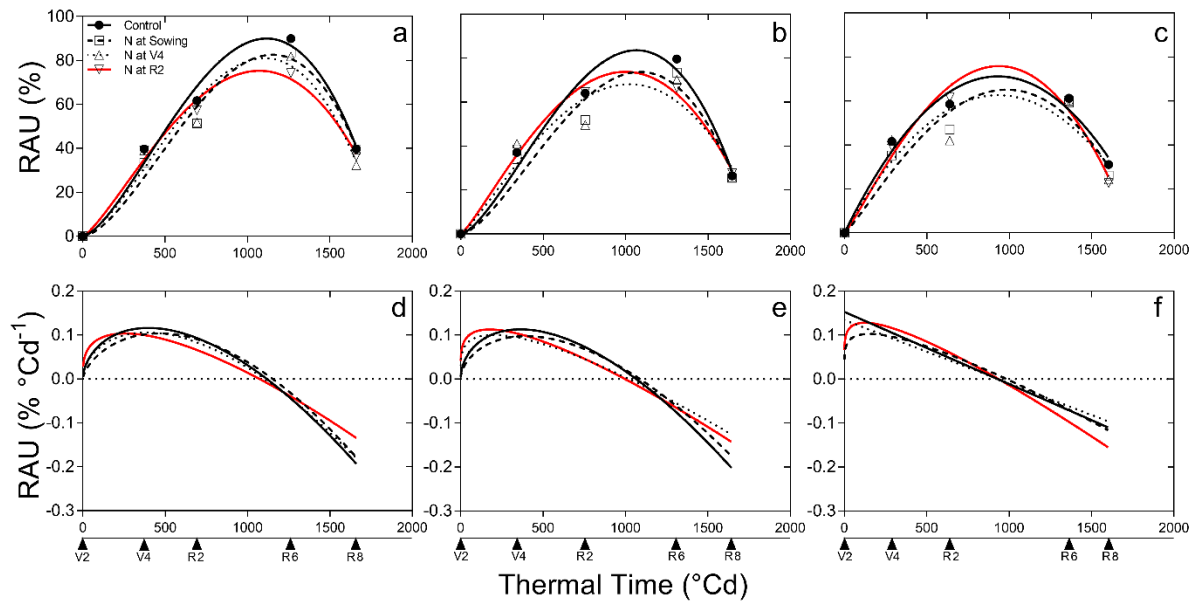


Figure 3.2: Seasonal changes in the proportion of the relative abundance of ureides (RAU) and RAU rate for the high (A,D), medium (B,E), and low (C,F) BNF groups for the control (black full line and black circles), N at sowing (broken line and empty squares), N at V4 (dotted line and empty triangles), and N at R2 (red full line and empty inverted triangles). Each point in V4 (fourth-leaf), R2 (full flowering), R6 (full seed), and R8 (full maturity) represents the average from the locations of each group.

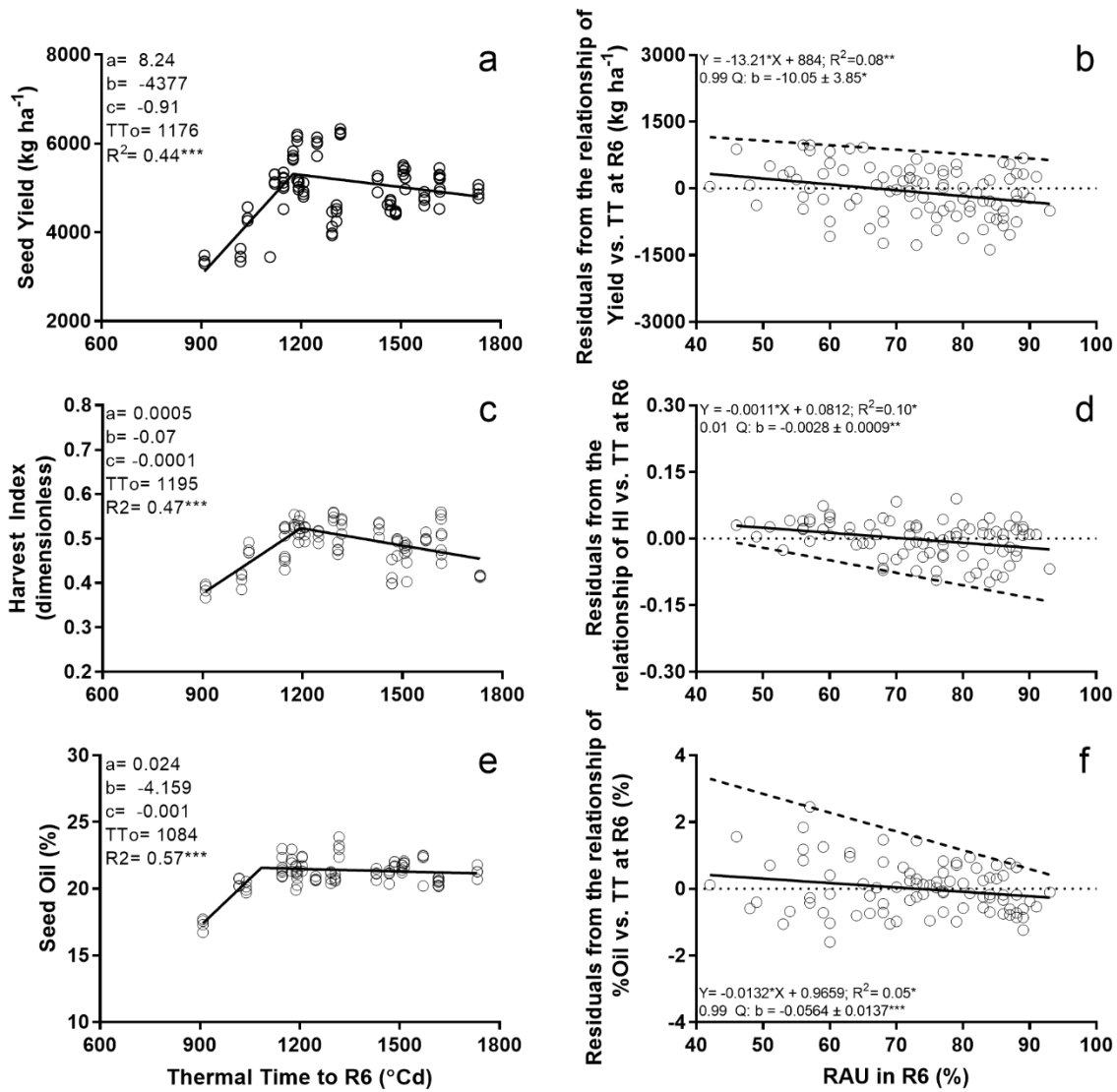


Figure 3.3: Relationship between seed yield (A), harvest index (C), seed oil concentration (E) and thermal time to R6. In (A, C, E), solid lines are bilinear models with fitted parameters a, b, c, TTo (Equation 2.5). Relationship between residuals from relationship in A (C), C (B), and E (F) with RAU at R6. Solid lines are least square regressions, and dashed lines are regressions for the 0.99 (b, f) and 0.01 (d) quantiles. Asterisks indicate significance of the coefficient: three asterisks, $P < 0.001$; two asterisks, $P < 0.01$; one asterisk, $P < 0.05$.

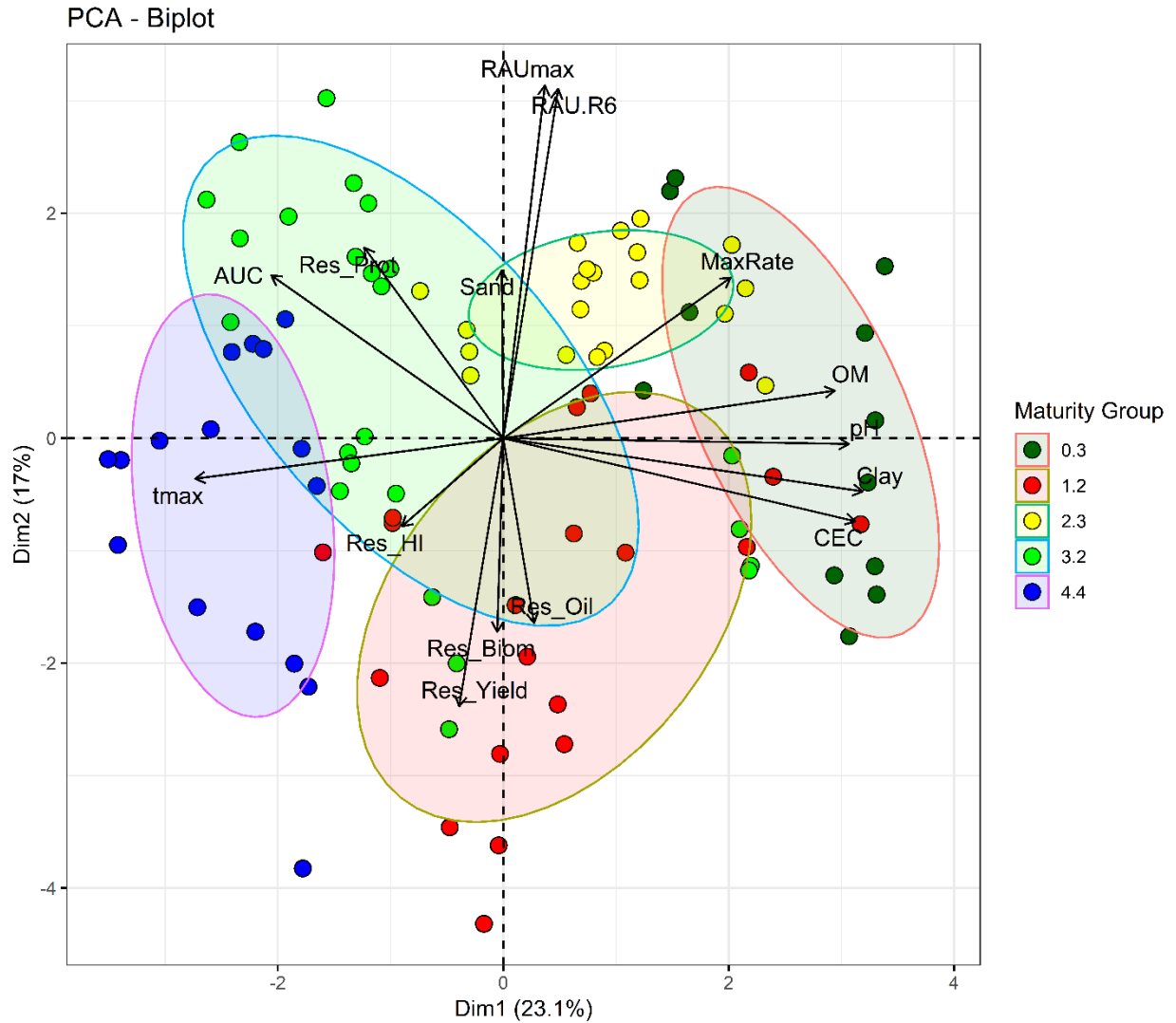


Figure 3.4: Principal component analysis of crop traits for each combination of treatment and maturity group. Concentration ellipses includes points each maturity group. Traits are maximum rate (MaxRate), residuals from seed protein concentration (Res_Prot), seed yield (Res_Yield), total biomass (Res_Biom), harvest index (Res_HI), oil concentration (Res_Oil), maximum RAU during the growing season (RAUmax), RAU at R6 (RAUR6), relative AUC (AUC), thermal time for RAUmax (tmax), soil organic matter (OM), soil pH, clay, sand, and cation exchange capacity (CEC) from each location.

Table 3.1: Location, latitude, longitude, variety, maturity group, sowing date, mean temperature (°C), cumulative water supply (rainfall plus irrigation; mm), vapor pressure deficit (VPD; kPa) from sowing to R7, primary tillage, soil texture (FAO; Land and Water Division, 2006), clay (%), sand (%), organic matter (OM; g 100 g⁻¹), pH and cation exchange capacity (CEC; cmol charge kg⁻¹ soil⁻¹) for soybean crops in the Midwest of US in 2016.

| Location | Latitude (N) | Longitude (W) | Variety | Maturity Group | Sowing date | Temperature | Water supply | VPD | Tillage | Soil Texture | Clay | Sand | OM | pH | CEC |
|---------------------------------|--------------|---------------|---------|----------------|-------------|-------------|--------------|------|------------|--------------|------|------|-----|-----|------|
| Attica (Ohio) | 41° 0' | 82° 48' | R2C3323 | 3.2 | 25-05 | 22.1 | 561 | 1.08 | Strip-Till | Medium | 20.5 | 11.4 | 2.0 | 6.5 | 15.0 |
| Beaver Dam (Wisconsin) | 43° 26' | 88° 53' | R2C1572 | 1.2 | 17-05 | 19.5 | 496 | 0.91 | Chisel | Medium | 22.5 | 7.2 | 4.0 | 6.7 | 21.5 |
| Blencoe (Iowa) | 41° 56' | 96° 5' | R2C3323 | 3.2 | 20-05 | 20.7 | 527 | 1.00 | Reduced | Fine | 47.5 | 5.5 | 2.5 | 7.9 | 38.5 |
| Britt (Iowa) | 43° 5' | 93° 52' | R2C2394 | 2.3 | 17-05 | 19.7 | 501 | 0.72 | Chisel | Medium | 15.0 | 50.0 | 7.0 | 7.8 | 12.1 |
| Clarksdale (Mississippi) | 34° 19' | 90° 29' | R2C4541 | 4.4 | 24-04 | 25.2 | 640 | 1.30 | Plow | Medium | 14.0 | 55.0 | 0.5 | 5.8 | 7.5 |
| Devils Lake West (North Dakota) | 48° 14' | 98° 53' | R2T0313 | 0.3 | 12-05 | 17.1 | 434 | 0.78 | Chisel | Fine | 23.0 | 40.0 | 4.0 | 7.0 | 19.9 |
| Effingham (Illinois) | 39° 9' | 88° 37' | R2C3323 | 3.2 | 24-05 | 22.8 | 580 | 0.93 | Chisel | Medium | 15.0 | 6.0 | 1.8 | 5.8 | 13.2 |
| Fargo (North Dakota) | 46° 44' | 96° 49' | R2T0313 | 0.3 | 25-05 | 19.7 | 501 | 1.04 | Chisel | Fine | 48.0 | 5.0 | 5.0 | 7.2 | 37.1 |
| Holloway (Minnesota) | 45° 14' | 95° 54' | R2C1572 | 1.2 | 05-05 | 18.3 | 465 | 0.97 | Chisel | Medium | 18.5 | 11.7 | 5.0 | 7.2 | 19.0 |
| Ithaca (Michigan) | 43° 15' | 84° 35' | R2C2394 | 2.3 | 02-06 | 19.3 | 490 | 0.94 | Chisel | Medium | 14.0 | 44.8 | 2.0 | 6.7 | 5.2 |
| Le Sueur (Minnesota) | 44° 28' | 94° 4' | R2C1572 | 1.2 | 16-05 | 19.5 | 497 | 0.88 | Chisel | Fine | 32.0 | 30.0 | 7.0 | 7.5 | 25.0 |
| Mayville (North Dakota) | 47° 17' | 97° 8' | R2T0313 | 0.3 | 11-05 | 17.9 | 454 | 0.89 | Chisel | Medium | 48.0 | 5.0 | 5.0 | 7.2 | 37.1 |
| Owensboro (Kentucky) | 37° 42' | 87° 11' | R2C4541 | 4.4 | 31-05 | 23.6 | 600 | 0.92 | Reduced | Medium | 13.0 | 9.0 | 2.0 | 6.2 | 11.6 |
| Pierre (South Dakota) | 44° 32' | 100° 27' | R2C1572 | 1.2 | 13-05 | 20.4 | 518 | 1.61 | Strip-Till | Fine | 22.0 | 9.4 | 3.0 | 6.4 | 18.9 |
| Pocahontas (Iowa) | 42° 43' | 94° 38' | R2C2394 | 2.3 | 18-05 | 19.8 | 502 | 0.92 | Chisel | Fine | 33.0 | 25.0 | 7.0 | 6.5 | 28.1 |
| Portland (Michigan) | 42° 53' | 84° 51' | R2C2394 | 2.3 | 03-06 | 20.0 | 507 | 1.02 | Strip-Till | Fine | 33.6 | 34.2 | 7.5 | 6.7 | 25.0 |
| Springfield (Illinois) | 39° 44' | 89° 45' | R2C3323 | 3.2 | 05-05 | 22.0 | 560 | 0.94 | Chisel | Medium | 32.0 | 1.7 | 4.5 | 6.0 | 26.9 |
| Thayer (Kansas) | 37° 33' | 95° 28' | R2C4541 | 4.4 | 10-05 | 23.0 | 583 | 1.04 | Strip-Till | Medium | 22.0 | 6.0 | 3.3 | 6.4 | 18.7 |
| Tipton (Indiana) | 40° 15' | 86° 2' | R2C3323 | 3.2 | 27-05 | 21.5 | 547 | 0.93 | Plow | Medium | 20.0 | 19.0 | 2.5 | 6.5 | 14.5 |
| Van Wert (Ohio) | 40° 48' | 84° 34' | R2C3323 | 3.2 | 27-05 | 20.9 | 531 | 1.08 | Strip-Till | Medium | 23.0 | 7.0 | 2.5 | 5.9 | 19.3 |
| Vincent (Iowa) | 42° 35' | 94° 1' | R2C2394 | 2.3 | 05-05 | 19.8 | 504 | 0.86 | Strip-Till | Fine | 30.0 | 31.0 | 6.0 | 6.2 | 25.6 |
| Wamego (Kansas) | 39° 12' | 96° 17' | R2C4541 | 4.4 | 13-05 | 22.2 | 564 | 1.27 | Strip-Till | Medium | 19.0 | 15.0 | 2.5 | 6.3 | 16.4 |
| West Salem (Wisconsin) | 43° 53' | 91° 6' | R2C1572 | 1.2 | 02-05 | 18.4 | 466 | 0.87 | Chisel | Medium | 17.5 | 13.6 | 2.5 | 6.2 | 15.2 |

Table 3.2: Seed yield (13.5% moisture), concentration of protein and oil in seed, and relative abundance of ureides at R6 (RAU_{R6}) for soybean crops in the Midwest of US. Nitrogen treatments are Control, N at sowing, N at V4, and N at R2. Averages for each site include the 95% confidence intervals. Sites are grouped in three classes: high BNF (> 75th quartile), medium BNF (25th-75th quartiles), and low BNF (< 25th quartile). Within each group, sites are ranked from high to low RAU_{R6}.

| Group | Site | Control | | | | N at sowing | | | | N at V4 | | | | N at R2 | | | | Average (n=12) | | | |
|--------|-------------|---------------------|-----------------------|------|-------------------|---------------------|-----------------------|------|-------------------|---------------------|-----------------------|------|-------------------|---------------------|-----------------------|------|-------------------|---------------------|-----------------------|-------------|-------------------|
| | | Seed Yield | Protein | Oil | RAU _{R6} | Seed Yield | Protein | Oil | RAU _{R6} | Seed Yield | Protein | Oil | RAU _{R6} | Seed Yield | Protein | Oil | RAU _{R6} | Seed Yield | Protein | Oil | RAU _{R6} |
| | | kg ha ⁻¹ | g 100 g ⁻¹ | % | | kg ha ⁻¹ | g 100 g ⁻¹ | % | | kg ha ⁻¹ | g 100 g ⁻¹ | % | | kg ha ⁻¹ | g 100 g ⁻¹ | % | | kg ha ⁻¹ | g 100 g ⁻¹ | % | |
| High | Devils Lake | 3349 | 40.2 | 17.3 | 93 | 3445 | 41.6 | 16.7 | 84 | 3482 | 40.0 | 17.7 | 85 | 3296 | 40.8 | 17.6 | 73 | 3393 ± 111 | 40.6 ± 6 | 17.3 ± 0.55 | 84 ± 6 |
| | Vincent | 5717 | 36.3 | 20.9 | 91 | 5997 | 36.2 | 20.7 | 87 | 6044 | 34.7 | 20.7 | 86 | 6137 | 35.7 | 20.6 | 89 | 5974 ± 169 | 35.7 ± 2 | 20.7 ± 0.38 | 88 ± 2 |
| | Pocahontas | 5023 | 37.1 | 21.1 | 90 | 5117 | 36.9 | 21.3 | 88 | 4964 | 35.6 | 20.8 | 88 | 5155 | 36.9 | 20.3 | 89 | 5060 ± 183 | 36.6 ± 2 | 20.9 ± 0.38 | 89 ± 2 |
| | Britt | 5348 | 36.8 | 20.9 | 89 | 5211 | 35.7 | 21.3 | 84 | 5118 | 37.1 | 21.0 | 83 | 5234 | 36.5 | 20.9 | 87 | 5228 ± 212 | 36.5 ± 2 | 21.0 ± 0.54 | 86 ± 2 |
| | Thayer | 5069 | 36.2 | 21.8 | 88 | 4855 | 37.7 | 21.3 | 74 | 4978 | 36.8 | 20.7 | 68 | 4773 | 38.7 | 21.3 | 42 | 4919 ± 285 | 37.3 ± 12 | 21.3 ± 0.47 | 68 ± 12 |
| | Tipton | 4614 | 40.4 | 20.6 | 88 | 4529 | 39.8 | 21.1 | 86 | 4247 | 40.9 | 20.8 | 80 | 4466 | 40.6 | 20.7 | 66 | 4464 ± 109 | 40.4 ± 6 | 20.8 ± 0.55 | 80 ± 6 |
| Medium | Fargo | 3344 | 38.7 | 20.7 | 87 | 3451 | 39.2 | 20.2 | 76 | 3634 | 38.1 | 20.8 | 68 | 3459 | 38.6 | 20.2 | 83 | 3472 ± 81 | 38.6 ± 6 | 20.5 ± 0.36 | 78 ± 6 |
| | Effingham | 4405 | 40.5 | 22.0 | 85 | 4435 | 39.9 | 21.7 | 86 | 4445 | 40.2 | 21.6 | 84 | 4478 | 38.6 | 22.0 | 79 | 4441 ± 343 | 39.8 ± 6 | 21.8 ± 0.46 | 84 ± 6 |
| | Attica | 4902 | 38.3 | 21.2 | 84 | 4902 | 38.1 | 21.1 | 83 | 5268 | 40.4 | 20.7 | 77 | 5206 | 38.7 | 21.5 | 80 | 5070 ± 159 | 38.9 ± 6 | 21.1 ± 0.36 | 81 ± 6 |
| | Owensboro | 4731 | 38.6 | 21.2 | 81 | 4618 | 36.5 | 21.1 | 86 | 4698 | 39.4 | 20.4 | 75 | 4471 | 39.5 | 21.4 | 76 | 4628 ± 137 | 38.5 ± 5 | 21.0 ± 0.48 | 80 ± 5 |
| | Portland | 4803 | 36.8 | 22.4 | 81 | 4921 | 37.1 | 21.7 | 78 | 5116 | 40.5 | 21.3 | 74 | 4856 | 38.9 | 22.3 | 56 | 4924 ± 148 | 38.3 ± 9 | 22.0 ± 0.74 | 72 ± 9 |
| | Mayville | 4260 | 35.1 | 19.9 | 80 | 4314 | 36.7 | 20.1 | 72 | 4263 | 36.8 | 19.7 | 64 | 4569 | 35.1 | 20.5 | 67 | 4352 ± 101 | 35.9 ± 5 | 20.1 ± 0.33 | 70 ± 5 |
| | Clarksdale | 5431 | 38.7 | 22.1 | 79 | 4965 | 37.4 | 21.9 | 82 | 5229 | 37.9 | 21.6 | 73 | 5229 | 37.6 | 21.9 | 71 | 5237 ± 326 | 37.9 ± 8 | 21.9 ± 0.68 | 76 ± 8 |
| | Wamego | 5448 | 37.2 | 20.2 | 79 | 4529 | 35.9 | 20.8 | 49 | 5298 | 37.3 | 20.3 | 70 | 5233 | 36.9 | 20.5 | 59 | 5127 ± 328 | 36.8 ± 10 | 20.4 ± 0.47 | 64 ± 10 |
| | Blencoe | 5466 | 35.6 | 21.2 | 77 | 5304 | 34.2 | 21.8 | 72 | 5513 | 35.2 | 21.4 | 75 | 5383 | 34.4 | 21.8 | 77 | 5416 ± 86 | 34.9 ± 4 | 21.5 ± 0.38 | 75 ± 4 |
| | Le Sueur | 4524 | 36.1 | 21.4 | 77 | 5042 | 34.5 | 22.4 | 77 | 4625 | 31.9 | 23.0 | 68 | 4996 | 36.2 | 21.6 | 70 | 4775 ± 351 | 34.7 ± 5 | 22.1 ± 0.69 | 73 ± 5 |
| Low | West Salem | 5826 | 37.4 | 23.0 | 73 | 5634 | 40.1 | 21.3 | 66 | 5667 | 39.6 | 22.2 | 51 | 5729 | 39.9 | 21.9 | 60 | 5714 ± 188 | 39.2 ± 6 | 22.1 ± 0.47 | 62 ± 6 |
| | Holloway | 5166 | 35.2 | 21.7 | 72 | 6150 | 35.7 | 21.7 | 65 | 6060 | 39.2 | 19.9 | 60 | 6204 | 36.9 | 21.2 | 57 | 5880 ± 415 | 36.7 ± 5 | 21.2 ± 0.61 | 64 ± 5 |
| | Beaver Dam | 5132 | - | - | 72 | 5304 | - | - | 62 | 5094 | - | - | 55 | 5307 | - | - | 72 | 5209 ± 232 | - | - | 65 ± 6 |
| | Springfield | 4911 | 38.4 | 20.2 | 69 | 4602 | 37.7 | 22.3 | 63 | 4784 | 36.5 | 22.4 | 56 | 4730 | 36.7 | 22.5 | 59 | 4757 ± 171 | 37.3 ± 8 | 21.9 ± 0.73 | 62 ± 8 |
| | Ithaca | 4128 | 36.9 | 21.3 | 60 | 3976 | 36 | 20.7 | 68 | 3934 | 36.0 | 21.2 | 73 | 4566 | 35.5 | 20.4 | 60 | 4151 ± 230 | 36.1 ± 8 | 20.8 ± 0.32 | 65 ± 8 |
| | Pierre | 6201 | 33.1 | 23.9 | 57 | 6232 | 33.8 | 23.0 | 46 | 6248 | 36.1 | 22.4 | 63 | 6326 | 34.5 | 23.3 | 56 | 6252 ± 259 | 34.4 ± 6 | 23.1 ± 0.47 | 56 ± 6 |
| | Van Wert | 4979 | 40.1 | 20.6 | 48 | 4913 | 40.4 | 20.8 | 57 | 5282 | 41.0 | 20.6 | 54 | 5204 | 41.8 | 20.2 | 53 | 5094 ± 130 | 40.8 ± 4 | 20.5 ± 0.46 | 54 ± 4 |

Table 3.3: Parameters \pm standard error of the evolution curves of RAU fitted for crops with high BNF (> 75th quartile), medium BNF (25th-75th quartiles), and low BNF (< 25th quartile). RAU_{max} is the peak RAU during the growing season (V2 to R7) reached at t_{max}, thermal time t_{0.5} when 50% of RAU_{max} is reached, the maximum rate reached at t_m, and the area under the curve AUC. Syx is the standard deviation of the residuals of the fitted curve, and R² is the coefficient of determination of the fitted curve (all p < 0.001).

| Group | Treatment | RAU _{max} | t _m | t _{max} | t _{0.5} | Maximum Rate | AUC | Syx | R ² |
|--------|-------------|--------------------------|----------------|------------------|------------------|---------------------|------------------|------|----------------|
| | | % | °Cd | °Cd | °Cd | % °Cd ⁻¹ | % °Cd | % | |
| High | Control | 90 \pm 5a ^a | 397 \pm 74 | 1117 \pm 29a | 473 | 0.116 | 92586 \pm 6648 | 12.8 | 0.85 |
| | N at Sowing | 83 \pm 5ab | 461 \pm 85 | 1153 \pm 34a | 514 | 0.104 | 82749 \pm 7417 | 13.8 | 0.81 |
| | N at V4 | 81 \pm 5ab | 388 \pm 78 | 1103 \pm 32b | 466 | 0.106 | 82619 \pm 6691 | 12.2 | 0.84 |
| | N at R2 | 75 \pm 6b | 257 \pm 125 | 1072 \pm 45c | 422 | 0.104 | 82142 \pm 9791 | 15.0 | 0.75 |
| Medium | Control | 84 \pm 3a | 362 \pm 48a | 1066 \pm 20a | 444 | 0.113 | 84986 \pm 6215 | 11.1 | 0.87 |
| | N at Sowing | 74 \pm 3b | 394 \pm 63a | 1089 \pm 26a | 464 | 0.098 | 75029 \pm 7208 | 12.8 | 0.78 |
| | N at V4 | 68 \pm 3cd | 199 \pm 87b | 1023 \pm 32ab | 365 | 0.101 | 75108 \pm 6375 | 13.1 | 0.75 |
| | N at R2 | 74 \pm 3bd | 176 \pm 71b | 999 \pm 27b | 349 | 0.113 | 80139 \pm 7394 | 11.7 | 0.82 |
| Low | Control | 71 \pm 7 | 0 \pm 187 | 931 \pm 58 | 272 | 0.153 | 78102 \pm 9439 | 14.9 | 0.71 |
| | N at Sowing | 65 \pm 7 | 152 \pm 164 | 985 \pm 54 | 337 | 0.103 | 68334 \pm 7577 | 14.0 | 0.68 |
| | N at V4 | 63 \pm 8 | 0 \pm 230 | 933 \pm 72 | 274 | 0.134 | 67982 \pm 7873 | 15.6 | 0.62 |
| | N at R2 | 76 \pm 6 | 125 \pm 114 | 936 \pm 42 | 313 | 0.128 | 77387 \pm 8160 | 12.2 | 0.80 |

Chapter 4 - Allometric analysis reveals enhanced reproductive allocation in historical set of soybean varieties

Under review in Field Crops Research

ABSTRACT

Seed yield is commonly expressed as the product of shoot biomass and harvest index (HI) at physiological maturity. However, HI is a size-dependent ratio unsuitable to describe shifts in reproductive partitioning in historical soybean [*Glycine max* (L.) Merr.] studies where selection has enhanced shoot biomass. The aim of this work was to analyze allocation of biomass to reproductive organs in the onset of seed filling (R5) using allometric analysis in a set of historical soybean varieties. Seven varieties released between 1980 and 2013 were evaluated in field trials in Rossville (Kansas, United States) under two N fertilizer rates (0 and 670 kg N ha⁻¹) in 2016 and 2017. Seed yield increased at a rate of 0.74 % yr⁻¹ (p<0.001); while the shoot biomass increased at a rate of 0.41 % yr⁻¹ (p<0.001), suggesting an increase in allocation to reproduction. However, the rate of increase in HI at maturity was not different from zero highlighting the inadequacy of this trait. Allometric exponents (slopes of the log-log relationships) relating pod and shoot biomass, pod and leaf, and pod and stem plant fractions increased linearly with the year of release (p<0.05). Allometric analyses thus revealed genetic gains in reproductive allocation not detected by HI at maturity. The latter outcome highlights the contribution of improved reproductive partitioning to soybean yield gains, and the need for allometric analyses to account for size-dependence in allocation of shoot biomass.

INTRODUCTION

Seed yield in crops is commonly expressed as the product between shoot biomass and the reproductive allocation through the HI (i.e., the ratio of seed to shoot (aboveground) biomass; Donald and Hamblin, 1976) both determined at physiological maturity. These two traits have been commonly used to dissect management and genotypic effects on yield improvement.

Reproductive allocation differs among cultivated species defining physiological strategies between shoot biomass accumulation and its allocation to reproductive organs, and is partially shaped by natural and agronomic selection and plant morphology (Vega et al., 2000, 2001a; Weiner et al., 2009). For example, the relationship between yield and crop or plant growth rate is linear in indeterminate crops such as soybean [*Glycine max* (L.) Merr.] and chickpea (*Cicer arietinum* L.) (Lake and Sadras, 2016; Vega et al., 2001a) and non-linear in maize (*Zea mays* L.) and sunflower (*Helianthus annuus* L.) where morphological constraints impose a ceiling in grain set (Vega et al., 2001a).

In soybean, the contribution of shoot biomass production to seed yield improvement has been large and consistent relative to the influence of HI in retrospective studies comparing historic sets of genotypes (Balboa et al., 2018; Rowntree et al., 2014; Specht et al., 1999). In contrast, the contribution of HI to genetic yield gain has been large and consistent in cereals (Hay, 1995; Slafer, 1994), with the exception of maize in North America, with HI presenting minimal changes over time, one percentage unit per decade (Duvick et al., 2004).

There are two main reasons for inconsistencies in shifts of soybean HI in comparisons of historic varieties. The first one is related to errors and different approaches in the estimation of HI, including intensity and timing of plant sampling (e.g., phenological stages), treatments applied, environmental conditions and genotypic background (Kumudini, 2002; Rowntree et al.,

2014). In particular, the loss of leaves in mature soybean is a major source of variation in HI (Hay, 1995; Schapaugh and Wilcox, 1980; Unkovich et al., 2010). The second is that the HI of soybeans is a size-dependent plant trait (Vega et al., 2000), and ratios are a biased measure of reproductive allocation when plant size varies. Thus, allometric analysis is needed to account for size-dependent variation in shoot biomass allocation (Jasienski and Bazzaz, 1999; Niklas, 1994; Pearsall, 1927; Poorter and Sack, 2012). Of special relevance for this paper, Qin et al. (2013) highlighted the bias and instability of HI in comparison to allometric exponents relating grain and vegetative biomass on log-log scales.

Soybean yield improvements are usually associated with seed number rather than seed weight (de Felipe et al., 2016). Seed set occurs between the beginning of flowering (R1) and the onset of seed filling (R5) where the number of reproductive sinks reaches a maximum (Egli and Zhen-wen, 1991). During this period, the rate of reproductive partitioning peaks and the relationship between crop growth rate and seed number is linear (Vega et al., 2001b) but with significant variation among environments and genotypes (Masino et al., 2018; Rotundo et al., 2012). Furthermore, other physiological frameworks include partitioning to reproductive structures and the seed set efficiency at R5 to explain seed number determination (Charles-Edwards, 1984). However, comparison of older and modern soybean varieties have shown similar dynamics in shoot biomass accumulation until R5 where newer cultivars portray greater rates of shoot biomass accumulation during the seed filling period (Shiraiwa and Hashikawa, 1995; Specht et al., 1999).

Historical shifts in biomass allocation patterns in soybean would benefit from an allometric perspective to account for size-dependent allocation. We propose to analyze allocation of biomass to reproductive organs in R5 using allometric analyses in a set of historical soybean

varieties. This evaluation will help to better understanding the effects of genetic gain in reproductive partitioning as a more accurate alternative to HI at physiological maturity.

MATERIALS AND METHODS

Crop husbandry

Field experiments were conducted in Kansas River Valley research station in Rossville, Kansas, United States (39°07′ N; 95°55′ W). Table 3.1 shows weather and soil details. Crops were sown after maize on May 12, 2016 and May 18, 2017 with seed inoculated using a commercial strain of *Bradyrhizobium japonicum* and commercially treated with insecticide and fungicide. Target plant density was 25.5 plants m⁻². Crops were rainfed and supplemented with irrigation when needed to avoid water stress. Pest and disease were chemically controlled as needed to avoid any potential yield losses. Weeds were chemically sprayed before sowing and manually removed during the season if needed.

Treatments and experimental design

We established a factorial experiment combining an historic set of seven varieties and two nitrogen rates over two growing seasons. Experiments were set in a split-plot design with three repetitions in a randomized complete block structure with N rates assigned to the main plots and varieties to sub-plots. Each plot was 10 m long with four rows separated at 0.76 m.

Varieties were: P3981 (released 1980, MG 3), 9391 (1987, MG 4), 9392 (1991, MG 3.8), 93B82 (1997, MG 3.8), 93B67 (2001, MG 3.8), 93M90 (2003, MG 3.9), and P35T58R (2013, MG 3) (Corteva Agriscience, Pioneer seeds; Johnston, IA). Three criteria were used to select these varieties: a narrow range of phenology, proven agronomic adaption, and wide adoption in

the Midwest productive area of the United States. To account for potential N-limitation in seed yield and biomass (Cafaro La Menza et al., 2017; Ortez et al., 2019), we established two N conditions: an unfertilized control and a high-N condition with 670 kg N ha⁻¹ of liquid urea ammonium nitrate (N–P–K, 28–0–0) evenly split in three applications at sowing, R1 (beginning flowering), and R3 (beginning pod) (Fehr and Caviness, 1977).

Measurements

Phenological stages were recorded twice a week using the scale of Fehr and Caviness (1977). Shoots were sampled at R5 and R8 (full maturity) stages. An area of 1.14 m² was sampled in one of the two central rows at each sampling time when at least 50% or more of the plants reached the targeted growth stage. Shoots were separated in stems (including petioles, herein termed as stem), pods (including seeds, herein termed as pods), and leaves. At R8, fallen leaves and petioles were collected together with the standing material in the sampled area. Samples were dried in a forced-air oven at 65°C until constant weight. Total aboveground biomass in R5 and R8 was calculated as the sum of the fractions and expressed in kg ha⁻¹. Seed yield was measured by harvesting the two central rows with a plot combine recording seed moisture at harvest. Individual seed weight was estimated from a sample of 100 dried seeds at harvest. Seed number per unit area was estimated using the individual seed weight and seed yield (both expressed on dry weight basis). Seed set efficiency was calculated as the ratio between seed number and reproductive biomass at R5. Harvest index was calculated as the ratio of seed yield to total aboveground biomass at R8.

Statistical analysis

To quantify the effect of genetic gain in crop traits mix linear models were fitted to test the effect of N rate across years of release considering the sources of variation of the experimental design. Nitrogen rate, year of release as continuous variable, and their interaction were used as fixed effects. Random effects included N rate nested in block and in the year effect. Models were fitted with the lmer() function in the ‘lme4’ package (Bates et al., 2015) for R software (R Core Team, 2018).

The rate of genetic change was calculated as the slope of the trait vs year of release fitted with ordinary least squares regression. Traits included seed yield, seed number, seed weight, shoot biomass at R8, harvest index, and allometric exponents (see below). For comparisons between selected traits we also calculated relative rate of genetic change using the newest cultivar as reference (Fischer et al. 2014) unless stated otherwise.

Allometric analysis

We fitted linear regressions in a log-log scale as proposed by Pearsall (1927):

$$\log W_A = \log \beta + \alpha \log W_B \quad \text{Equation 4.1}$$

where W is dry weight, subscripts A and B indicate plant parts, $\log \beta$ is the intercept coefficient and the slope α is the allometric exponent representing the ratio between logarithmic growth rate of parts A and B. For the objectives of this study, we emphasized the allometric exponent to probe for putative shifts in dry matter allocation at R5. We focused on R5 because the number of reproductive sinks peaks at this stage (see Introduction), and a large proportion of biomass has

already been allocated to reproduction, close to 70% of the total accumulated at maturity (Bender et al., 2015; Gaspar et al., 2017), and leaf dry matter can be measured reliably.

For each variety, we calculated the allometric exponents α relating (a) the dry matter of each organ vs. the total shoot biomass excluding that organ to avoid spurious correlations (Brett, 2004; Coleman et al., 1993; Weiner, 2004) and (b) the pair-wise relationships between organs. Data were log-transformed and Equation 4.1 was fitted using the reduced major-axis (RMA) analysis (Ebert and Russell, 1994) to account for the error of both measured variables. The function `sma()` from the ‘`smatr`’ package (Warton et al., 2012) for R software was used to test significance ($H_0: \alpha \neq 0$) and 95% confidence interval of slopes. All coefficients are reported in Table B.1.

RESULTS

Seed yield genetic gain

Experimental seasons showed similar growing conditions for soil characteristics, temperature, water supply, and accumulated evapotranspiration (Table 4.1). Growing conditions were reflected in similar average seed yield of 2988 ± 100 in 2016 and 2969 ± 89 kg ha⁻¹ in 2017.

Genetic gain for seed yield was 33.1 kg ha⁻¹ yr⁻¹ (Fig. 3.1) or 0.74 % yr⁻¹ (Fig. 4.3) irrespective of N rate. The seed yield vs year of release regressions were parallel, with an offset of 461 kg ha⁻¹ associated with nitrogen rate. Seed number per m² increased at 23 seed m⁻² yr⁻¹ (Fig. 4.2A) or 0.65 % yr⁻¹ (Fig. 4.3) irrespective of N rate. Seed weight remained constant across years with an average of 147 ± 2 mg seed⁻¹ at high nitrogen and 139 ± 2 mg seed⁻¹ at control

(Fig. 4.2C). Thus, seed size explained the N-driven offset in the yield versus the year of release regressions.

Shoot biomass at R8 ranged from $8270 \pm 713 \text{ kg ha}^{-1}$ for the oldest variety (P35T58R) to $10249 \pm 651 \text{ kg ha}^{-1}$ in the newest one (P3981), and increased linearly with year of release at $56.9 \text{ kg ha}^{-1} \text{ yr}^{-1}$ (Fig. 4.2B) or $0.41 \% \text{ yr}^{-1}$ (Fig. 4.3). Harvest index ranged from 0.29 to 0.42 and showed no trend with year of release (Fig. 4.2D).

Reproductive allometry in R5

Biomass in R5 ranged between 4177 and 5987 kg ha^{-1} , with no association with year of release ($P > 0.05$) or response to nitrogen ($P > 0.05$; data not shown). Seed set efficiency averaged 33 seed g^{-1} with no variation with N or year of release.

All allometric exponents were significantly different from zero (Figure B.1; Table B1). The allometric exponent relating pod and shoot biomass in R5 increased linearly with the year of release (Fig. 4.4E) revealing a greater relative increase in reproductive to plant biomass in modern varieties. Furthermore, 95% confidence intervals for the allometric exponent " α " were around 1 in older varieties (i.e., similar relative growth rate between pod and plant) and were greater than 1 in newer varieties, revealing a shift in relative growth in favor of reproductive structures. Allometric exponents from the relationships pod versus leaf and pod versus stem increased linearly with year of release (Fig. 4.4D and F). Both relationships reinforce the analysis in Fig. 4.4E and reflect larger relative growth to reproductive organs in relation to stem and leaf, with a relative increase of 1.27 and 1.43 $\% \text{ yr}^{-1}$, respectively.

Allometric exponents relating stem and shoot biomass, leaf and shoot biomass (Fig. 4.4A and C), and stem and leaf (Fig. 4.4B) lacked association with year of release. In all these

relationships, allometric exponent remained not significant from " α " = 1, highlighting a conserved balance between stem and leaf.

DISCUSSION

Seed yield increased linearly for a set of seven commercial varieties released between 1980 and 2013. The overall increase of 33.1 kg ha⁻¹ yr⁻¹ in both N conditions of this study is in agreement with those present in the literature (de Felipe et al., 2016; Rowntree et al., 2013; Rincker et al., 2014; Specht et al., 1999). In common with these studies, genetic gain in seed number was the driver of genetic gain in crop yield.

Wilson et al. (2014) found the rate of genetic gain in 57 varieties (released from 1923 to 2007) was 27 kg ha⁻¹ yr⁻¹ for crops grown under 570 kg N ha⁻¹ compared to their non-fertilized counterparts that increased at 23 kg ha⁻¹ yr⁻¹. In contrast, we found the rate of genetic gain was independent of N, but N caused an offset in the time-trends associated with larger seed weight in fertilized crops.

In our study, shoot biomass at maturity increased with year of release, with no time-trend for HI. However, the relative rate of change in biomass was only a fraction of the relative rate of change in yield (Fig. 4.3). To explore if this is particular for our set of varieties, we compared the relative rate of change in yield and biomass from independent studies. In all cases, the relative rate of improvement in biomass was smaller than the relative rate of improvement in yield (Fig. 4.5). This analysis reinforces three conclusions: biomass explains only partially the change in yield, improved reproductive partitioning is a necessary part of the improvement in yield, and that HI is unsuitable to capture shifts in partitioning to grain.

Findings across literature (i) agree with ours in the increase in biomass at maturity in modern cultivars, (ii) overlook the question if these changes in biomass fully account for yield gains (Fig. 4.5), and (iii) are contradictory in regards to HI contribution with reports of no HI trends (Cregan and Yaklich, 1986; De Bruin and Pedersen, 2009; Frederick et al., 1991) or positive trends in HI (Balboa et al., 2018; Specht et al., 1999; Suhre et al., 2014). In addition to the differences in HI calculations, the overall interpretation of allocation ratios is ambiguous when biomass has increased with year of release and allocation is size-dependent.

Allometry of reproductive allocation shifted in varieties released between 1980 and 2013. Time-trends of allometric exponents portrayed allocation of biomass in favor of reproductive organs in modern varieties with stable relative growth between stem and leaf (Fig. 4.4). We did not find association between biomass at R5 and year of release in agreement with literature (Shiraiwa and Hashikawa, 1995; Specht et al., 1999). De Bruin and Pedersen (2009) documented first differences in biomass production at R5.5 stage (~7 days after R5). Despite small differences, the above-mentioned studies agreed that greater biomass accumulation occurs during the seed filling period. Some of the physiological traits contributing to greater biomass accumulation during this period include greater stomatal conductance (Morrison et al., 1999), less plant lodging (de Felipe et al., 2016; Voldeng et al., 1997), and a higher photosynthetic rate with smaller leaf area (Jin et al., 2010; Morrison et al., 1999). Consistently, we found greater dry matter allocation into pods was at the expense of leaf biomass in newer varieties (Fig. 4.4D).

New varieties also had higher allocation to pod relative to stem (Fig. 4.4F). This may be partially associated with both reduced plant height in newer varieties (Morrison et al., 2000) and a greater seed yield on main stems associated with more pods per nod (Suhre et al., 2014). Same

authors reported greater ability for branching in newer varieties when soybean plants are not crowded, whereas in higher densities commonly used for soybean, differences were not evident.

We found no changes in leaf: stem allometric exponents with selection for yield or in response to nitrogen and season. This is consistent with allometric analyses on a broad spectrum of seed plant species showing isometric scaling in annual growth among vegetative organs (Niklas and Enquist, 2002a, 2002b). These studies also provide strong evidence that vegetative growth is size-independent, consistent with our findings (Fig. 4.4B).

Genetic variation for allometric relationships of biomass allocation determine different reproductive strategies (Weiner, 2004). This is relevant in the context of crop production and plant breeding. Contrasting physiological strategies has been reported for different species using conceptual frameworks to explain seed number in maize (Tamagno et al., 2015) and soybean (Rotundo et al., 2012). In both cases, genotypes clustered based on the arrangement of different combinations of plant growth and ratios of reproductive partitioning and seed set efficiency. Our results demonstrate that patterns in reproductive allocation can be detected with allometric analyses and thus define reproductive strategies with potential breeding applications. Qin et al. (2013) highlighted the risks of using HI as a selection criterion if allometry is not taken into account.

Uncertainties on the effectiveness of selecting for HI in soybean have risen due to the difficulty in phenotyping large number of genotypes (Schapaugh and Wilcox, 1980) and other physiological constraints associated with greater nutrient demand from seeds (Lawn, 1989; Tamagno et al., 2017). To the best of our knowledge, studies analyzing shifts in seed yield or biomass allocation in soybean varieties have never been approached with the allometric

perspective. Agronomic studies using ratios such as HI would benefit from its interpretation when complemented with allometric relationships (Poorter and Sack, 2012).

CONCLUSION

For our set of varieties and environments, seed yield in soybean increased at a rate of 0.7 % yr⁻¹ between 1980 and 2013 whereas the rate of increase in shoot biomass was only 0.4 % yr⁻¹. New analysis of independent studies reinforced the conclusion that improvement in biomass explains only part of the genetic gain in yield. This means allocation to seed must have increased, but HI did not capture this necessary change in allocation. Allometric analysis at the end of the seed set revealed unambiguous increase in relative allocation into pods at the expense of leaf and stem.

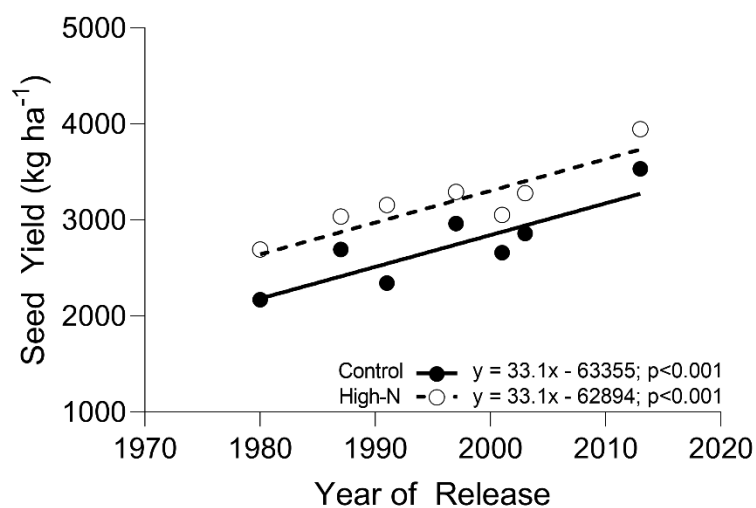


Figure 4.1: Relationship of seed yield with year of release. Full circles are controls and open circles are high-N condition. Solid and dashed lines are the regressions for the control and high-N condition, respectively. Coefficient of determination (R^2) of the full model including N condition, year of release, and its interaction is 0.44.

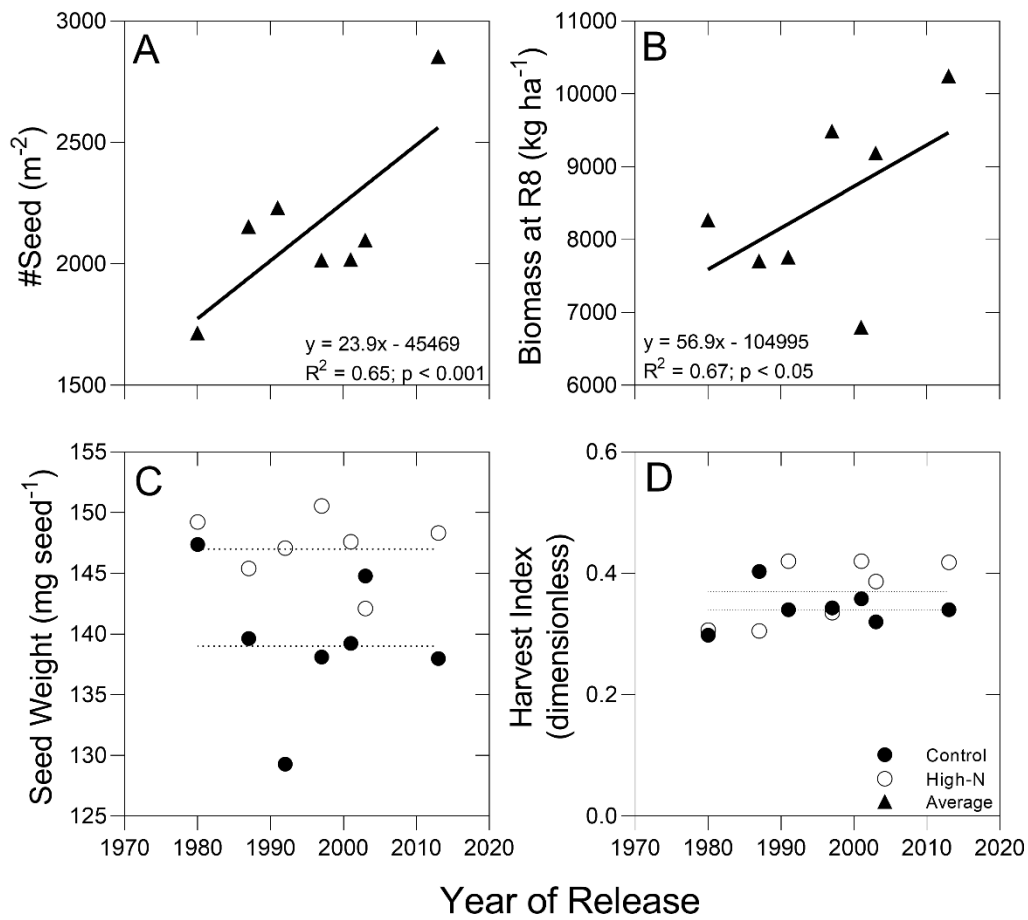


Figure 4.2: Seed number (A), biomass at R8 (B), seed weight (C), and harvest index (D) with year of release. Full circles are controls and open circles are high-N condition. Triangles are the overall mean when the analysis was significant only for year of release. Dotted lines in (C) and (D) are averages for each N condition.

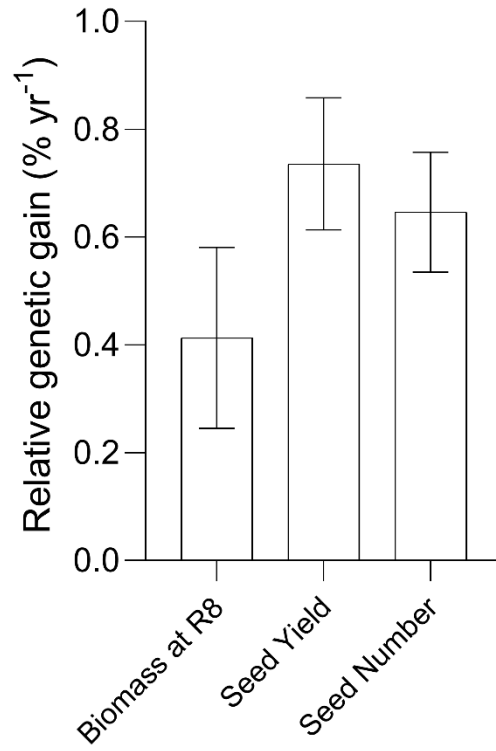


Figure 4.3: Relative genetic gain (\pm s.e.) expressed in percentage (%) per year for biomass at R8 (aboveground, shoot biomass), seed yield, and seed number.

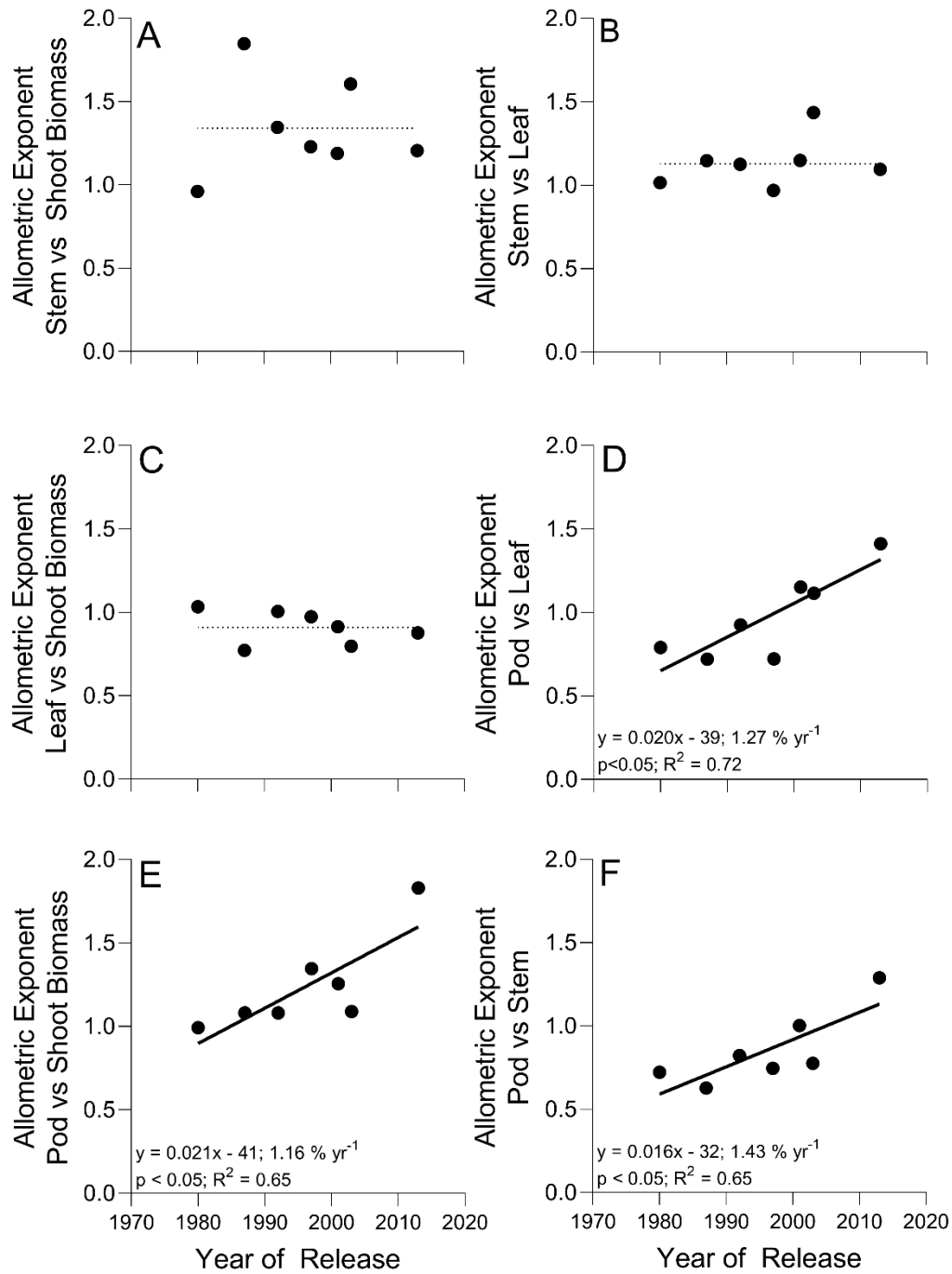


Figure 4.4: Relationship between allometric exponent from relationships between stem and shoot biomass (excluding stem; A), stem and leaf biomass (B), leaf and shoot biomass (excluding leaf; C), pod and leaf biomass (D), pod and shoot biomass (excluding pod; E), and pod and stem (F) with year of release for seven soybean varieties released between 1980 and 2013. Solid lines are the regressions and dotted lines are the average when regressions were not significant.

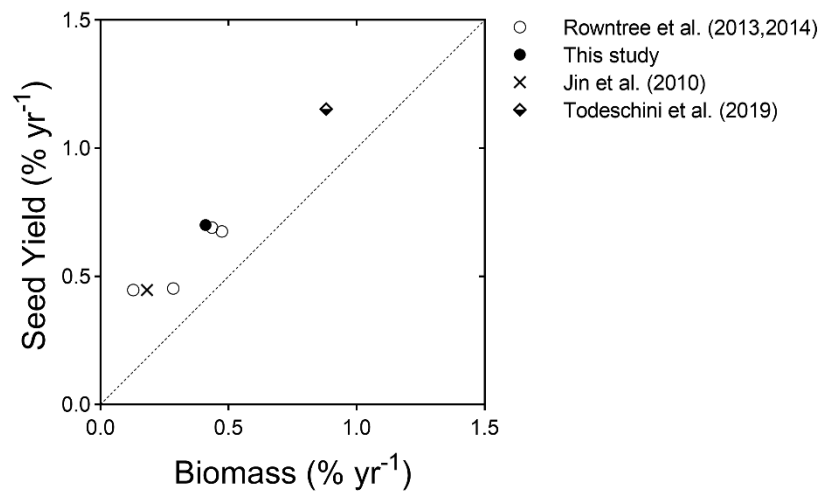


Figure 4.5: Comparison between relative rate of increase in seed yield and shoot biomass across the literature and this study. Dashed line is the 1:1 relationship. Relative rates were estimated dividing the absolute rate of genetic gain and the predicted value of the newest variety reported in each study for either seed yield or biomass (Boerma, 1979).

Table 4.1: Mean temperature, solar radiation, water supply, cumulative reference evapotranspiration (ET_o) and soil characteristics for experiments in Rossville (Kansas, United States) over two seasons (i.e., from sowing to harvest). Water supply is rainfall plus irrigation. Soil characteristics measured at sowing include pH, cation exchange capacity (CEC), organic matter (OM), N-nitrates (N-NO₃; measured at 60cm soil depth), phosphorous (Mehlich-P), potassium (K), calcium (Ca) and magnesium (Mg) measured in the 30 cm-top soil.

| Season | Temperature | Solar Radiation | Water Supply | ET _o | Soil pH | CEC | OM | N-NO ₃ | Mehlich-P | K | Ca | Mg |
|--------|-------------|-------------------|--------------|-----------------|---------|-------------------------|-----|-------------------|-----------|---------------------|------|-----|
| | °C | MJ/m ² | mm | mm | | meq 100 g ⁻¹ | % | | | mg kg ⁻¹ | | |
| 2016 | 23.0 | 19.6 | 795 | 653 | 6.9 | 11.0 | 2.2 | 3.0 | 21 | 153 | 2074 | 202 |
| 2017 | 22.5 | 20.1 | 744 | 659 | 7.3 | 5.8 | 1.3 | 2.7 | 13 | 90 | 951 | 95 |

Chapter 5 - Dynamics of oil and fatty acid accumulation during seed development in historical soybean varieties

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ABSTRACT

Soybean seed oil concentration and fatty acid (FA) composition are relevant traits defining oil quality and economic value for industry. In spite of the importance of FA composition for soybean value, a detailed description of FA profile and accumulation during seed filling in contrasting soybean varieties and management conditions is currently lacking. This study aims to: i) evaluate whether genetic progress in soybean yield indirectly affected the FA profile, ii) assess the influence of N fertilization on the FA profile, iii) characterize the temporal dynamics of FA accumulation during seed filling, and iv) evaluate the relative contribution of FA accumulation rate vs. duration on final FA seed content. Seven soybean varieties released between 1980 and 2013 were field grown under control (0 kg N ha⁻¹ added) and fertilized (670 kg N ha⁻¹ added) treatments. In addition to seed yield, total seed oil and FA contents during seed filling were recorded by sampling seeds from the beginning of seed formation to maturity. Genetic progress was evident in seed yield with an increase of 31.3 kg ha⁻¹ year⁻¹. Total oil concentration (g kg⁻¹) slightly decreased across year of release for the control treatment, respectively. Fatty acid concentration (g 100 g⁻¹) profile was not associated with year of release but affected by varieties. Variation in total oil and FA contents (mg seed⁻¹) across varieties and N treatments was mostly associated to longer filling but was also affected by accumulation rates. Interestingly, individual FA concentration at maturity was independent from FA content. Our results indicate past breeding efforts have not modified soybean FA profile. The fact that changes in FA contents were not translated in changes in FA concentration indicates

that seed control of FA profile is narrow. Disentangling the physiological mechanism associated with FA homeostasis warrants future investigations.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is one of the major sources of vegetable oil worldwide. The FA composition of soybean oil is a key determinant of oil value and end-use. Genetic progress in soybean yield has been already documented as an outcome of commercial breeding (de Felipe et al., 2016; Rowntree et al., 2014). However, reports of indirect effects of breeding on FA profile are currently lacking in the scientific literature. From a historical perspective, studies analyzing changes in FA concentration (or content) are limited and have emphasized changes in total oil concentration (Jin et al., 2010; Rowntree et al., 2013; Voldeng et al., 1997).

Different FA offers diverse functionality to the use of soybean oil. The five major FAs in soybean oil are palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3). The current vegetable oil market demands high oleic content for feed, oil, and biodiesel industries due to increased heat stability and low oxidation capacity. Linoleic and linolenic acids are essential FAs for human diet. The ratio between mono (oleic) and polyunsaturated (linoleic + linolenic) acids (herein termed as OI/Li+Ln) is a general indicator of oil quality (Carrera and Dardanelli, 2017; Gao et al., 2009). Fatty acid composition at maturity is the end result of metabolic pathways highly regulated by environmental and management conditions during the seed filling period (Carrera et al., 2009, 2011; Gao et al., 2009; Scilewski da Costa Zanatta et al., 2017) and genetic background (Bellaloui et al., 2009; Ishikawa et al., 2001; Chapman and Ohlrogge, 2012). While these effects were evaluated when analyzing final FA profiles, a detailed

description of the effect of genetics and management on temporal dynamics of FA accumulation is lacking.

Fatty acid concentration is commonly expressed relative to total seed oil content (g FA 100 g oil⁻¹, Gerde and White, 2008). Therefore, at a seed level, changes in an individual FA concentration might be related to variation in that particular FA content (mg FA seed⁻¹), variation in another FA, and/or variation in total oil content (mg oil seed⁻¹). Ultimately, similar concentrations of a specific FA can be achieved with different contents of that particular FA in the seed. This concept was used to dissect physiological strategies to increase seed protein concentration in seeds with reduced seed oil and carbohydrates (Poeta et al., 2016). This idea highlights the importance of understanding how the end FA concentration is attained based on final FA contents per seed. As any component in the seed, the final FA content per seed can be expressed as the product of the rate of accumulation and the duration of the seed filling (Egli, 1998). This period is usually divided into three phases: (i) the initial lag phase, when active cell division occurs and cell structures are formed; (ii) the effective filling period (EFP), when seed growth rate peaked; and (iii) the late maturation phase, when maximum seed dry mass was achieved (Black and Derek Bewley, 1994). Dissecting the physiological determinants of each FA content is important to better understand possible developmental limitations to FA final concentration.

The current study has four objectives. The first objective was to evaluate whether genetic progress of soybean yield indirectly affected the FA profile. Complementarily, variety effects, regardless of genetic progress, were assessed to quantify genotypic variability in oil and FA concentration. The second objective was to determine the influence of N fertilization on FA profile. The third objective was to characterize the temporal dynamics of FA accumulation

during seed filling. This objective will quantify the seed developmental parameters, duration and rate of accumulation, controlling final content of oil and each particular FA. The four and last objective was to evaluate the relative contribution of FA accumulation rate vs. duration on final FA seed content. This objective will allow testing the influence of final FA content on final FA concentration. To address these objectives a two-year field experiment evaluating seven soybean varieties released between 1980 and 2013 was carried out under control (0 kg N ha⁻¹ added) and fertilized (670 kg N ha⁻¹ added) treatments.

MATERIALS AND METHODS

Field experiments and plant measurements

Field studies were conducted at the Kansas River Valley research station in Rossville, Kansas, United States (39°07′ N; 95°55′ W) during 2016 and 2017 growing seasons. Planting dates were 12 May 2016 and 18 May 2017. The plot size was 10 m long with four rows spaced at 76 cm. For all treatments, seeds were inoculated and plots were maintained weed- and pest-free during the growing season. Both years were planted in corn-soybean rotations. Seven soybean varieties from Corteva Agriscience (Johnston, Iowa, US) released between 1980 and 2013 were evaluated: P3981 (1980), 9391 (1987), 9392 (1991), 93B82 (1997), 93B67 (2001), 93M90 (2003), and P35T58R (2013). Genotypes are representative of each year of release and were widely grown in the Midwest region of the United States.

Varieties were sown under rainfed conditions and received irrigation when necessary to avoid water stress (Table 5.1). A weather station recorded daily temperature, radiation, relative humidity, and precipitation. Vapor pressure deficit was calculated using maximum daily temperature to quantify atmospheric demand during the seed filling period (Howell and Dusek,

1996). Soil samples at 15 cm depth were collected before sowing to quantify pH, organic matter, cation exchange capacity, phosphorous (Mehlich-P) concentration. Nitrate-N concentration was measured at 60 cm depth. Soil descriptors are presented for each site-year, with corn as the previous crop.

Varieties were tested under two contrasting N treatments: a control, without N fertilization and a high-N, with 670 kg N ha⁻¹ equally split at planting, R1 (beginning flowering), and R3 (beginning pod) stages (Fehr and Caviness, 1977). The high-N treatment aims to provide a non-limiting N growing environment with the purpose of preventing N deficiencies (Cafaro La Menza et al., 2017). Fertilizer was applied with a hand-held backpack sprayer in each plot, utilizing liquid urea ammonium nitrate (UAN; N–P–K, 28–0–0) as the fertilizer source. Plots were arranged in a split-plot design with complete randomized blocks with varieties as subplots and the N scenarios as main plots, all replicated three times.

For seed filling determination, pod samples were collected weekly after the onset of seed filling (R5) until maturity from an area of 0.23 m² (4-6 plants). Pods were removed from the first and last four nodes to avoid differences in developmental and growth stages and in order to obtain a representative measure at the plant level (Poeta et al., 2014). Individual samples comprised from 40 to 80 seeds, weighed to calculate individual seed dry weight after drying at 65° C until reaching constant weight. At harvest, two center rows from each plot were harvested with a plot combine. Seed yield is expressed in a seed dry basis.

Total oil and fatty acid content determination

Four dry seeds were powdered and total lipids content alongside an internal standard (500 µg triheptadecanoin) transmethylated by adding 3 mL of 2.5% (v/v) H₂SO₄/methanol heating at

80°C for 1h. After cooling, the fatty acid methyl esters were extracted by adding 2 mL of water and 3 mL hexane and quantified by gas chromatography using a DB-23 column (Agilent J & W GC column, Agilent Technologies, Santa Clara, CA). The oven temperature was initially 200°C for 2 min; then ramped to 240°C at 10°C min⁻¹ and held at that temperature for 4 min.

Chromatogram peaks were identified by comparison with a 37 component standard mix (Supelco # 47885-U) and areas were corrected for flame ionization detector response and oil content determined as described previously (Li et al., 2006). Fatty acids content (palmitic, stearic, oleic, linoleic, and linolenic) and total oil for each sample were estimated as the product between seed dry weight (mg seed⁻¹) and component concentration. Concentrations of oleic acid and linoleic plus linolenic were used to calculate the Ol/Li+Ln ratio.

Changes in total oil and FA content from the beginning of seed filling to maturity were modeled comparing using the Weibull growth function (Ratkowsky, 1983; Equation 4.1) describing a sigmoidal pattern with an asymptotic plateau:

$$\text{Component (mg seed}^{-1}\text{)} = W_{\text{max}} - \text{Drop} \times \exp[-\exp(\text{lrc}) \times \text{TT}^{\text{pwr}}] \quad \text{Equation 5.1}$$

where TT is thermal time after R5 growth stage in degree days (°Cd; base temperature = 8°C; Sinclair et al., 2003), W_{max} is the maximum weight, Drop is the difference between the minimum and maximum weights, lrc the natural log of a rate constant and pwr the power to which TT during the SFP is raised. Fatty acid concentration in seeds was characterized by fitting quadratic and cubic equations for stearic, oleic, and linoleic and one-phase decay function for palmitic and linoleic:

$$\text{Fatty acid (g FA 100 g}^{-1}\text{)} = (\text{Y0-NS}) \times \exp^{-k \times \text{TT}} + \text{NS} \quad \text{Equation 5.2}$$

where Y_0 and NS are the value of FA concentration in the beginning and at the end of seed filling, respectively, and k is the rate constant. All parameters from Equation 5.1 and 5.2 are reported in the supplementary material (Tables C.1, C.3, and C.4).

The maximum rate of accumulation was obtained as the maximum point of the derivative function of Equation 5.1. The EFP is the maximum content of the seed component divided to the maximum growth rate (Egli, 1998). The intersection between the projection of the linear curve of maximum rate with the maximum content predicted by the function was defined as the end of the EFP following Egli (1998).

Statistical analysis

Traits evaluated for genetic gain were analyzed using linear mixed-effects models in R (R Core Team, 2017; version 3.5.1, lme4 package, lmer function) (Bates et al., 2015). The year of release was used as a continuous numerical variable, N treatment, and its interaction with the year of release were considered fixed effects whereas block and year of the study as a random effect. The N treatment factor was nested within blocks and year of the study factors. Differences between varieties were analyzed using analysis of variance model using variety, N treatment, and their interaction as fixed effects. Same model assumptions were used for random and nested effects.

Models to predict the changes in seed components during the seed-filling period were fitted using Equation 5.1 and analyzed using nonlinear mixed effects models (nlme package, nlme function) (Pinheiro et al., 2017). The significance of variety, N treatment, and their interaction was tested following a stepwise approach by fitting all possible combinations of factors and comparing models using the Akaike Information Criterion (AIC) between the

resulting models (Table 5.3). Lastly, to make inferences on the fixed effects on each parameter of the model, all possible combinations of fixed effects were tested and selected based on the AIC for the parameters (Tables C.1 and C.2). In each combination, main factors and their interactions were considered as fixed effects and year and block as random effects. The N treatment was nested within blocks and years factors.

RESULTS

Genetic progress in seed yield and associated changes in oil and fatty acid concentrations

Genetic progress for soybean yield was observed at a rate of $33.1 \text{ kg ha}^{-1} \text{ yr}^{-1}$ under both N treatments ($p < 0.001$; Fig. 5.1A). High N treatment out-yielded the control across years of release with a constant yield difference of 461 kg ha^{-1} as predicted in the model ($p < 0.01$). Total seed oil concentration was significant only for the interaction year of release by N treatment ($p < 0.05$; Fig. 5.1B). The control treatment showed a decrease in oil concentration at a rate of $0.33 \text{ g kg}^{-1} \text{ yr}^{-1}$, whereas with N fertilization the oil concentration remained constant.

Palmitic, oleic, and linoleic acid seed concentrations were neither affected by year of release nor by N ($p > 0.05$; Fig 5.1C, E, G). Although stearic and linolenic did not show a significant trend with the year of release, their concentrations significantly improved when N was added by 5.4 and 4.0% in stearic and linolenic, respectively. The oleic to polyunsaturated FA ratio did not change with the year of release (Fig. 5.1H).

Variety effects on seed yield, oil, and fatty acid concentration

Differences among varieties were significant in all traits except total seed oil concentration (Table 5.2). Main effects of variety ($p < 0.001$) and N treatment ($p < 0.01$) were significant for seed yield (Table 5.2). Varieties showed a range of yield ranging from 2752 to 3742 kg ha⁻¹, with an increase in seed yield by 14.4% due to N relative to the control. Total seed oil concentration ranged between 199 and 224 g kg⁻¹ among varieties without showing significant effects from any of the evaluated factors.

All FAs presented significant differences among varieties but differing in their magnitude (Table 5.2). Palmitic acid ranged only 4% between minimum and maximum across all soybean varieties. Stearic acid was the only main FA displaying small but significant ($p < 0.05$) decrease in its concentration from 3.84 g FA 100 g oil⁻¹ in the high-N to 3.63 g FA 100 g oil⁻¹ in the control. Oleic and linoleic concentrations were significant across varieties ($p < 0.05$), ranging from 5 to 11% from the minimum to maximum average values, respectively. Varieties differed significantly for the linolenic concentration ($p < 0.001$), with the variety 93M90 showing a tighter value of 8.07 g FA 100 g oil⁻¹ from the rest ($p < 0.05$). In contrast to the time-trend analysis in Fig. 5.1, differences between N treatments were not detected here. The mono to polyunsaturated FA ratio was also significant among varieties ($p < 0.05$) but with no impact due to N or their interaction.

Temporal dynamics of seed oil and FA accumulation

Nonlinear models for total oil and all main FAs were all significant for main effects of variety and N treatment as supported by AIC selection criteria (Table 5.3). Similarly, the parameter W_{\max} was significant for the same factors in all seed components (Table C.1). High N

increased the content of total oil and all the FAs ($p < 0.05$; Table 5.4). Total oil content increased by an average of 4.6 mg seed^{-1} in the N-fertilized while also showing considerable variation among varieties ranging from 26.8 to $34.6 \text{ mg seed}^{-1}$ (Fig 5.2; Table 5.4). The variety 93B82 displayed the highest values of total oil content as well as FA with the exception of linolenic (Table 5.4).

The saturated FAs stearic and palmitic showed an overall increase by 13 and 17%, respectively, with the addition of N relative to the control ($p < 0.05$; Table 5.4). Oleic acid was the fatty acid displaying the least genetic variability for content (narrowed variation) with negligible differences among varieties. Overall, polyunsaturated FA also showed differences among varieties and N treatments (Fig. 5.4, 5.5; Table 5.4), but with contrasting dynamics in their accumulation. Linoleic acid concentration depicted a quadratic increase during the seed-filling period without showing differences between N scenarios (Figure C.4); whereas linolenic acid concentration depicted a dilution curve with significant interaction between variety and N treatment (Figure C.5).

Seed filling rate and duration affecting final FA content

The final content of any seed component is determined by the accumulation rate and duration of the effective filing period (EFP). Total oil content was positively related to EFP duration ($p < 0.001$; Fig. 5.6A) but not with the rate of accumulation (Fig. 5.6B). Palmitic, stearic, and oleic contents were all significantly related to EFP duration and accumulation rates (Fig. 5.6C-H); however, in all cases variation in seed contents were largely explained by EFP duration (R^2 s 0.50-0.65) rather than by accumulation rates (R^2 s 0.39-0.48). In all cases, EFP duration was shorter for stearic acid relative to palmitic (Fig. 5.3) showing different FA

dynamics during the seed-filling period. Linoleic and linolenic acid were both strongly associated with changes in EFP duration ($p < 0.001$; Fig. 5.6I and K) whereas rate did not show significant effects on the changes in FA content. Duration of the EFP accounted for 62 and 78% of the variation in linoleic and linolenic content, respectively.

Concentration and content of seed components

The concentration and content of total oil and all FA showed consistent genetic variability in this study (Tables 5.2 and 5.4). Comparison between seed content and concentration reflects the differential response of varieties to achieve different seed composition (Fig. 5.7). Differences among varieties and treatments using 95% confidence intervals depict similar seed concentrations through a wide range of final contents. For instance, linoleic acid concentration in the genotype 93B67 was 51.3 and 52.4 g FA 100 g oil⁻¹ for the control and the high N treatment, respectively, whereas the seed content was 12.7 and 15.1 mg seed⁻¹ in each N treatment, respectively. This represents 2% of the change in concentration versus 16% of variation in the content. Similar trends were documented for other genotypes and other seed components.

DISCUSSION

Fatty acid profile in soybean seeds is highly linked to genetics and environmental conditions during seed filling (Medic et al., 2014). Cultivar differences in seed FA concentration (g FA 100 g oil⁻¹) were not associated with year of release or N treatment (only stearic decreased in high N) (Table 5.3). This indicates that, at least for the set of historical soybean varieties tested here, breeding for increased yield had no influence on seed FA profile. On the contrary, seed FA

content (mg seed^{-1}) consistently increased in response to N (Table 5.4). In general, variation in seed FA content was greatly accounted for a longer EFP ($R^2 > 0.50$ in all cases; Fig. 4.6) even when smaller contributions of the rate were significant (Fig. 5.6). These results indicate different degrees of independence in the rate and duration for each component during the seed filling. For instance, saturated and monounsaturated FA contents were associated with both accumulation rate and EFP duration whereas polyunsaturated FA increased their content only when EFP was longer (Fig. 5.6). To the best of our knowledge, this is the first study reporting seed accumulation parameters of individual FA accumulation in-planta under field conditions. The kinetic parameters of temporal dynamics of FA accumulation in seeds can guide the parametrization of metabolic models for tailoring specific process to obtain desired FA profiles.

Interaction of the year of release with N treatment was weak but significant for total oil concentration (Fig. 5.1) showing a decrease of 0.1 \% yr^{-1} . This effect can be argued to be biologically and agronomically not relevant. Trends in total oil concentration reported in the literature have been inconsistent. Some reports indicated no changes in oil associated to year of release while others showed increases (Rincker et al., 2014; Wilson et al., 2014, de Felipe et al., 2016; Jin et al., 2010).

The lack of correlation between seed FA concentration and content (Fig. 5.7) suggests that there are different biochemical pathways to attain similar FA profiles. This also indicates that for impacting any particular seed FA concentration, the content of that specific FA needs to be increased in several orders of magnitude. For example, the increase in oleic concentration from 22% in the soybean wild type to 85% in the FAD-2 downregulated transgenic varieties was associated with an increase in oleic seed content from 6 to 24 mg seed^{-1} (Spear et al., 2013; Graef et al. 2009). Therefore, the lack of correlation between FA concentration and content we

observed might only hold in situations of reduced variation in FA contents as associated with native genetic variation and management and/or environmental conditions.

It is likely that increases in oil and FA contents due to longer EFP are related to greater soil N supply, delaying leaf senescence (visually observed), and plausibly maintaining carbon supply to reproductive sinks. Seed oil accumulation relies on current carbon assimilation and translocation from other organs in the plant during seed filling (Sehgal et al., 2018). Therefore, oil accumulation is sensitive to reductions in carbon supply at this stage (Rotundo and Westgate, 2010; Turner et al., 2005). Studies using labeled carbon confirmed the greater contribution of current assimilated carbon in leaves and fruits (i.e., pod walls and seeds; Allen et al., 2009; Ruuska, 2004) to lipid synthesis in later stages of seed development (Yamagata et al., 1987). From an N nutrition standpoint, soil-derived N assimilation during seed filling can contribute up to 50% of total N uptake during this period (Córdova et al., 2019; Zapata et al., 1987) potentially delaying leaf senescence and extending photosynthetic activity (Ryle et al., 1978).

Variation between concentration and content were evidently larger for the latter in all seed components (Fig. 5.7) even though there were variety effects in all FAs concentration (Table 5.2). These results emphasize that increases in content of a particular FA in seeds do not warrant a higher concentration. Despite differences among varieties, this pattern of response in concentration and content could indicate a certain degree of homeostatic control of seed FA. Seed homeostasis has also been shown in soybean mutants with silenced genes for storage proteins (Kinney et al., 2007; Takahashi et al., 2003). Through regulations in the proteome soybean mutants compensated seed protein with other types of proteins resulting in protein concentration homeostasis (Schmidt et al., 2011). However, it is important to remark that environmental boundaries of this study did not explore extreme conditions such as water

limitation (Carrera et al., 2009) or extreme temperatures (Dornbos and Mullen, 1992) during the seed filling period that can drive higher ranges of variation in FA concentration. Therefore, the variation in FA reported in this study is relevant within the context of genotype by environment by management (G x E x M) tested but wider variations in those factors relative to the dynamic in FA during the seed filling period requires further attention.

CONCLUSIONS

We explored shifts in seed FA concentration in a set of historical soybean varieties released between 1980 and 2013. Total oil concentration was negatively associated with the year of release in the control but displayed a more stable over time trend when N was applied. Changes in FA concentrations and Ol/Li+Ln ratio were neither related to the year of release nor with N treatment. However, variety variability was observed in FA concentrations. Greater N supply increased the content (mg seed⁻¹) of total oil and FA in the same magnitude regardless of the soybean varieties (and year of release) evaluated in this study. Longer duration of the EFP was the main driver underpinning increases in FA content. However, palmitic, stearic, and oleic content depicted a certain level (in a lower extent) of association with the rate of accumulation. Fatty acid concentration was independent of variations in FA content. The extent to which this lack of association occurs requires more attention to expose new pathways and to guide the parametrization of metabolic models to obtain desired FA profiles.

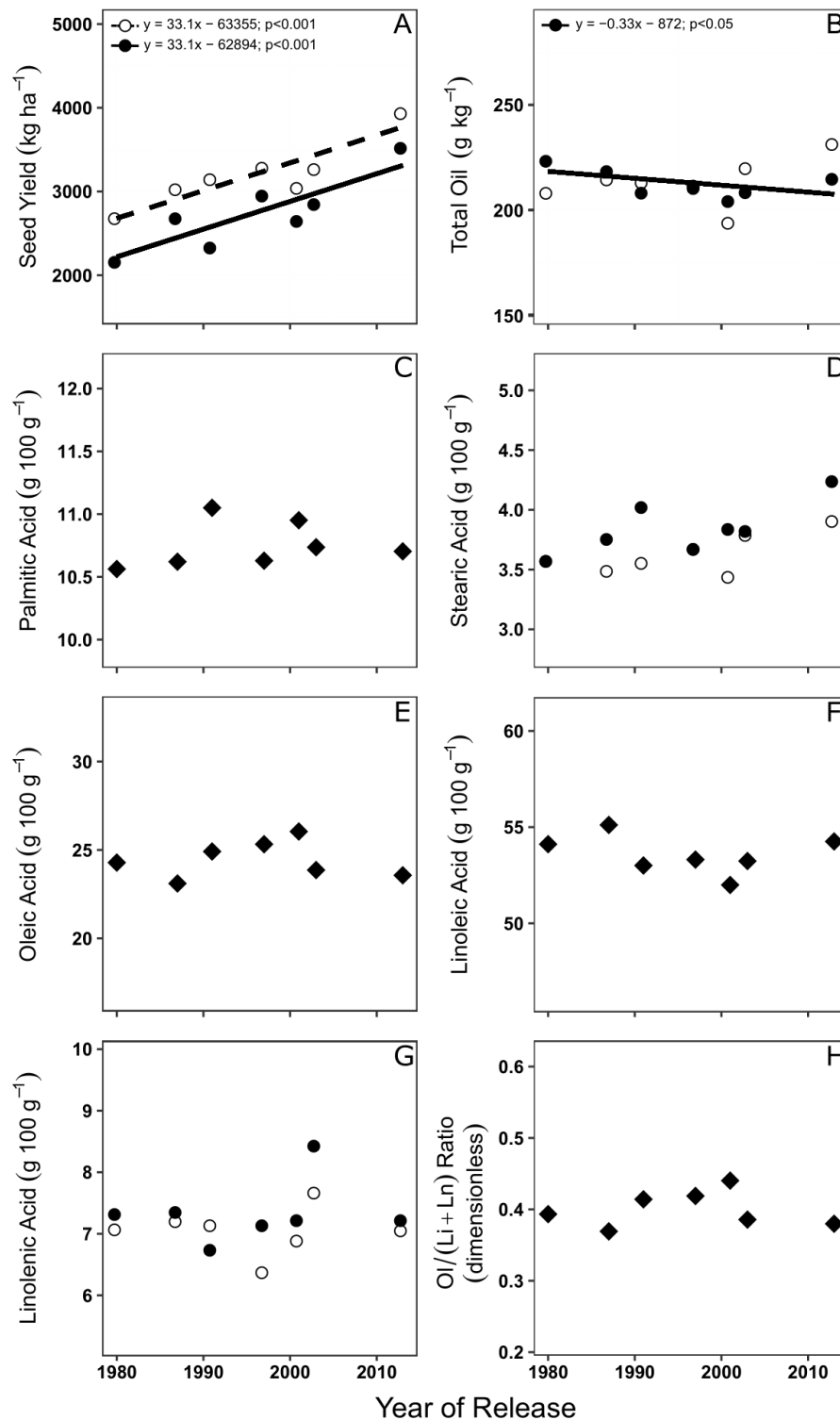


Figure 5.1: Relationship between the year of release and seed yield (A), total oil (B), palmitic (C), stearic (D), oleic (E), linoleic (F), linolenic (G), and the Ol/Li+Ln ratio (H). Open circles are the high N treatment, full black circles are the control, and diamonds represent average values when differences for source of variation were not significant.

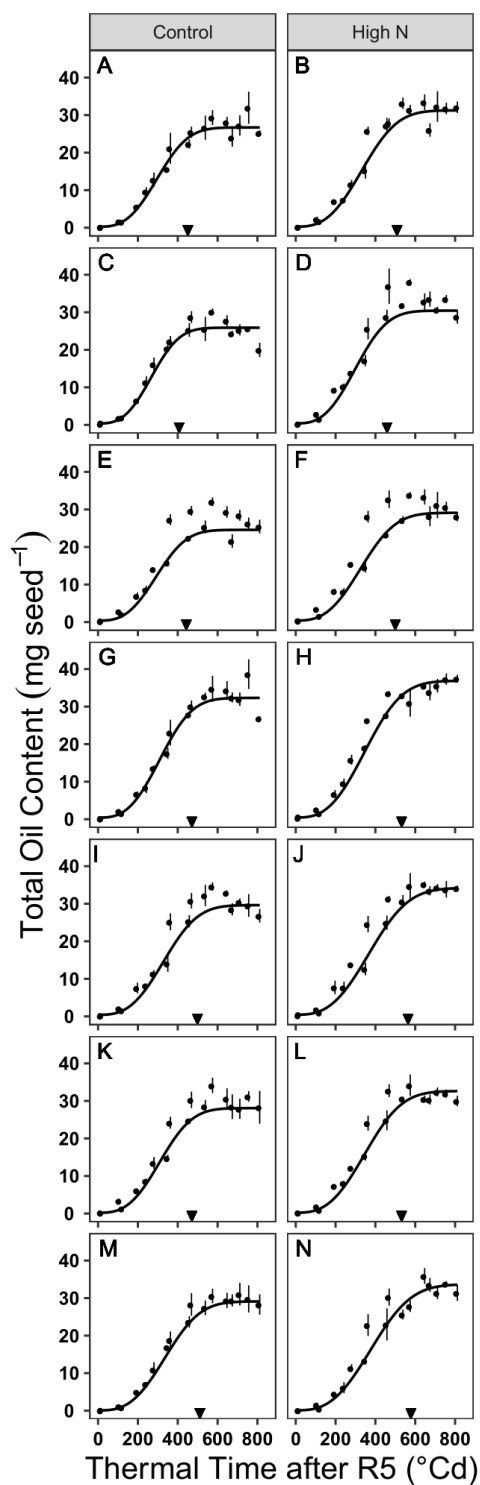


Figure 5.2: Changes in total oil content in the control and high-N treatment for 9391 (A-B), 9392 (C-D), 93B67 (E-F), 93B82 (G-H), 93M90 (I-J), P35T58R (K-L), and P3981 (M-N) variety. Each data point represents the average value of the three blocks in one year. Vertical bars represent the standard error. Triangles in the independent variable represent the end of the EFP.

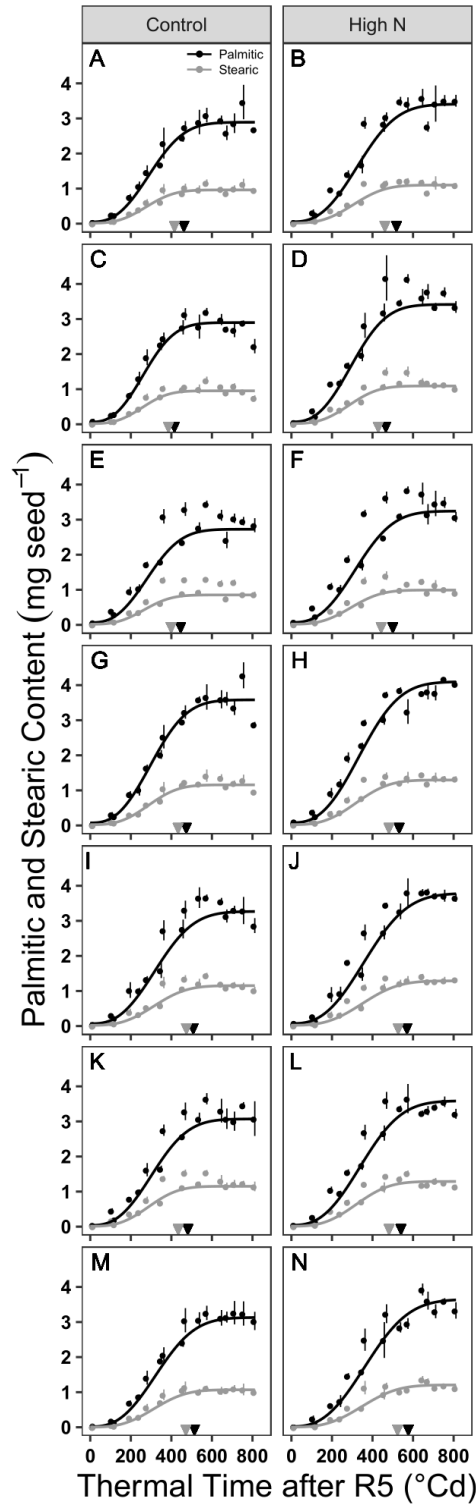


Figure 5.3: Changes in palmitic acid and stearic acid content in the control and high-N treatment for 9391 (A-B), 9392 (C-D), 93B67 (E-F), 93B82 (G-H), 93M90 (I-J), P35T58R (K-L), and P3981 (M-N) variety. Each data point represents the average value of the three blocks in one year. Vertical bars represent the standard error. Triangles in the independent variable represent the end of the EFP.

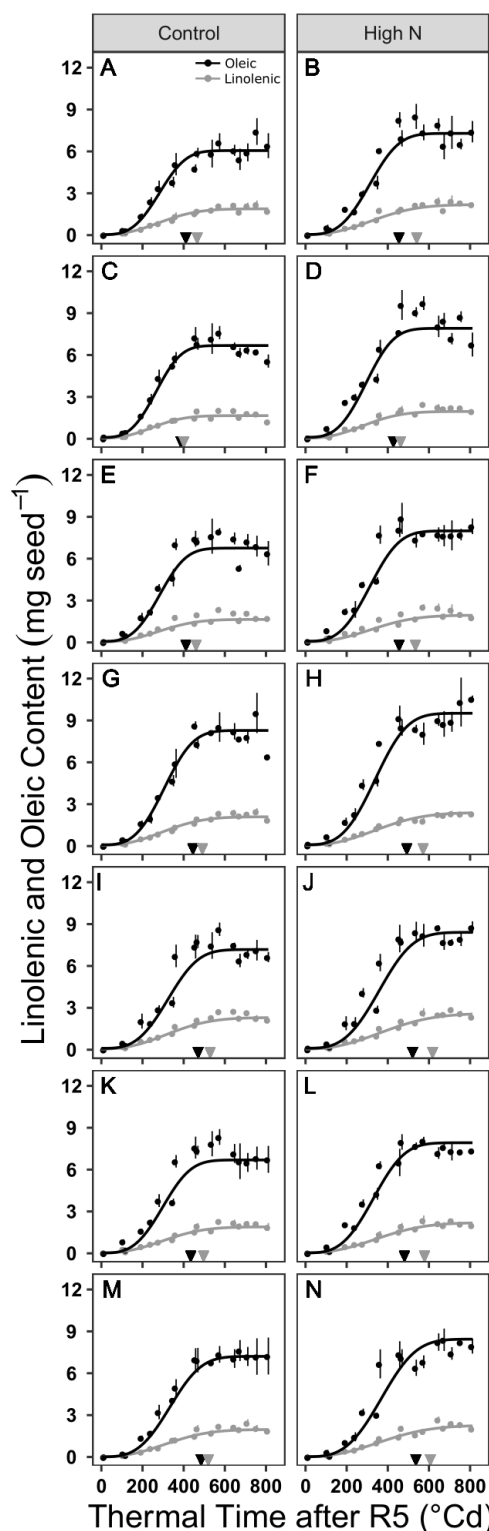


Figure 5.4: Changes in linolenic acid and oleic acid content in the control and high-N treatment for 9391 (A-B), 9392 (C-D), 93B67 (E-F), 93B82 (G-H), 93M90 (I-J), P35T58R (K-L), and P3981 (M-N) variety. Each data point represents the average value of the three blocks in one year. Vertical bars represent the standard error. Triangles in the independent variable represent the end of the EFP.

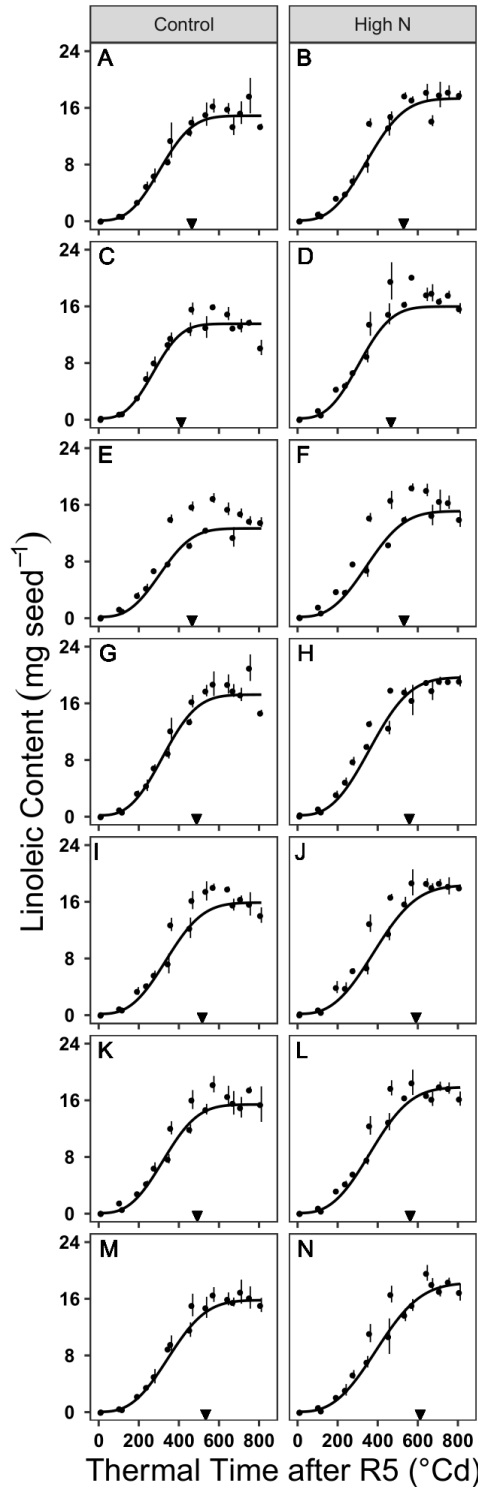


Figure 5.5: Changes in linoleic content in the control and high-N treatment for 9391 (A-B), 9392 (C-D), 93B67 (E-F), 93B82 (G-H), 93M90 (I-J), P35T58R (K-L), and P3981 (M-N) variety. Each data point represents the average value of the three blocks in one year. Vertical bars represent the standard error. Triangles in the independent variable represent the end of the EFP.

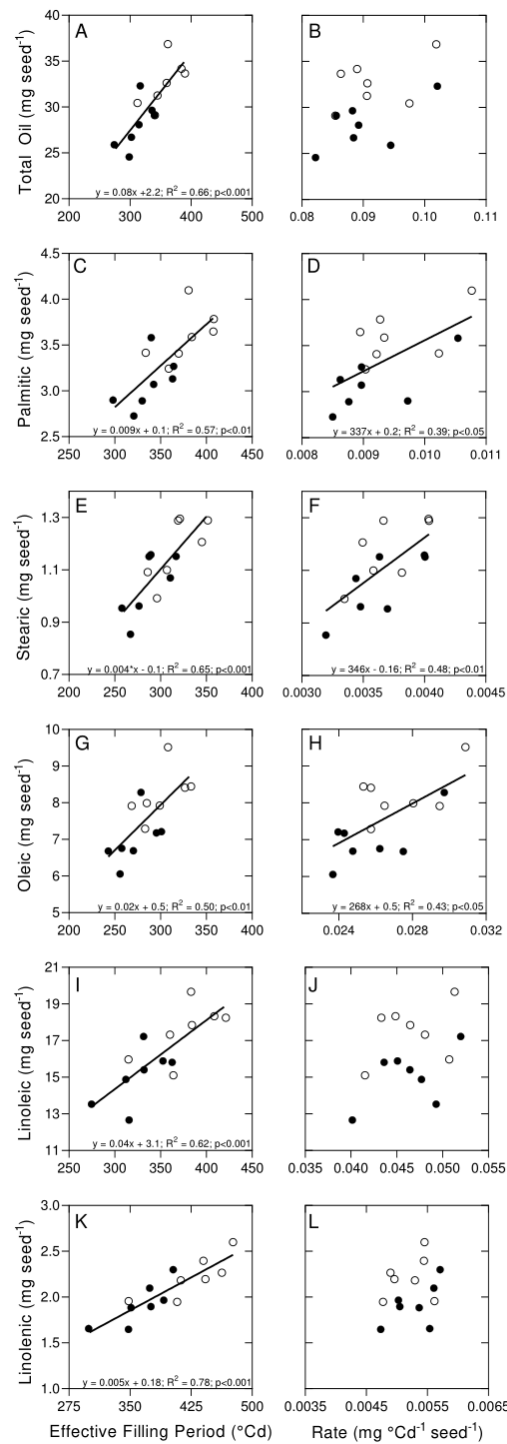


Figure 5.6: Relationship between total oil, palmitic, stearic, oleic, linoleic, and linolenic acids with EFP duration (A, C, E, G, I, K) and the rate of accumulation (B, D, F, H, J, L). Full black circles represent the control treatment and open circles the high-N treatment. Each data point represents the mean value of the observed component content and the predicted EFP and rate from models fitted in Fig. 4.2-5.

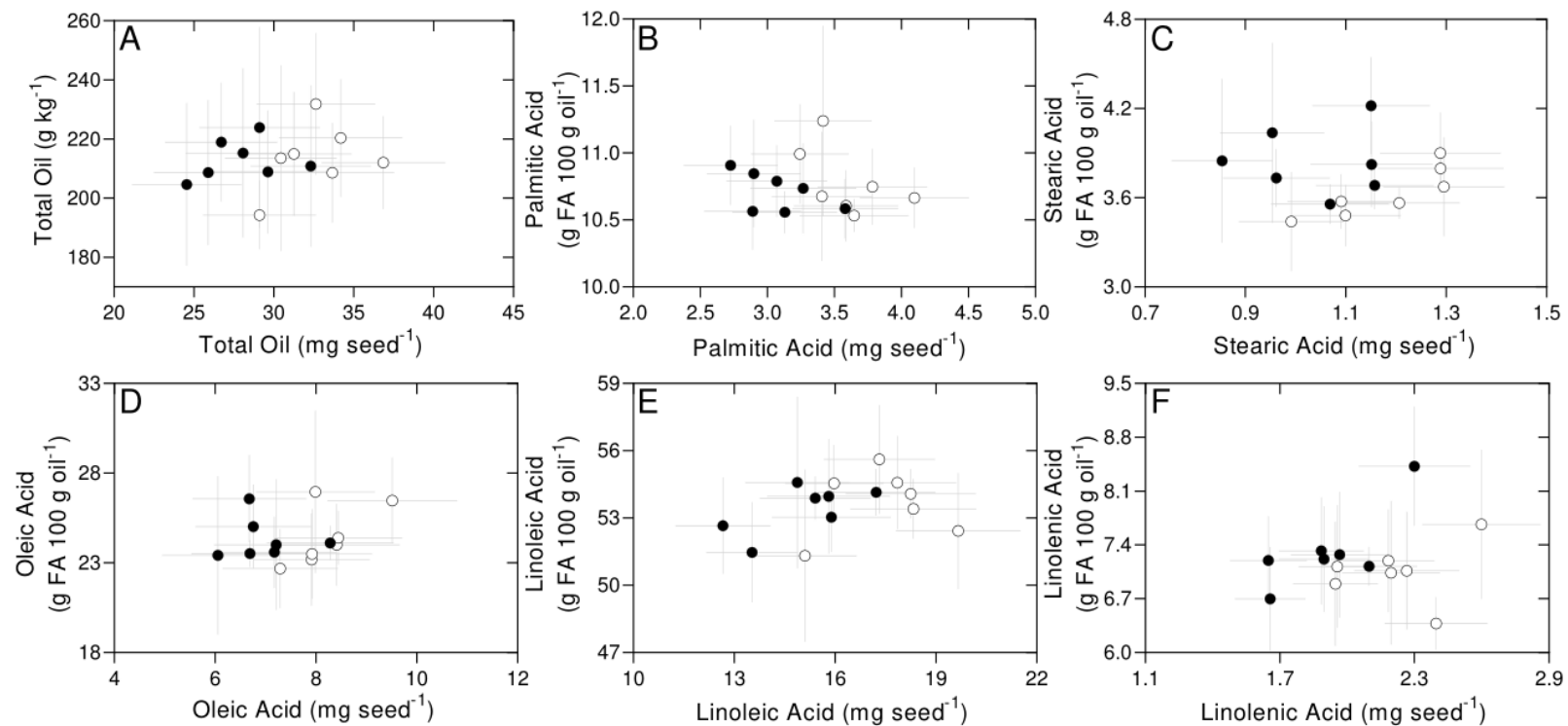


Figure 5.7: Comparison between seed concentrations against the content of total oil, palmitic, stearic, oleic, linoleic, and linolenic acids. Vertical and horizontal bars represent the confidence interval of the mean values for content and concentration. Full black circles represent the control treatment and open circles the high-N treatment.

Table 5.1: Temperature, solar radiation, and vapor pressure deficit (VPD) between R5-R8, water supply (rainfall plus irrigation) for the whole growing season, soil pH, cation exchange capacity (CEC), N-nitrate, phosphorous (P), and potassium (K) for the 2016 and 2017 seasons.

| Year | R5-R8 | | | Water Supply | Soil pH | CEC | OM | NO3-N | P | K |
|------|-------------|--------------------|------|--------------|---------|-------------------------|-------------------|-------|---------------------|-----|
| | Temperature | Solar Radiation | VPD | | | | | | | |
| | °C | MJ m ⁻² | kPa | mm | | meq 100 g ⁻¹ | g g ⁻¹ | | mg kg ⁻¹ | |
| 2016 | 21.8 | 16.9 | 0.99 | 795 | 6.9 | 11.0 | 2.2 | 3.0 | 21 | 153 |
| 2017 | 21.2 | 15.8 | 0.96 | 744 | 7.3 | 5.8 | 1.3 | 2.7 | 13 | 90 |

Table 5.2: Mean values and ANOVA for seed yield, total oil, palmitic, stearic, oleic, linoleic, linolenic seed concentration, and the Ol/Li+Ln ratio for each variety and N treatment.

| Variety | N Treatment | Seed Yield | Total Oil | Palmitic | Stearic | Oleic | Linoleic | Linolenic | [Ol]/[Li+Ln] |
|-----------------------|-------------|---------------------------------|--------------------|------------------------------|--------------------|--------------------|--------------------|-------------------|--------------------|
| | | kg ha ⁻¹ | g kg ⁻¹ | g FA 100 g oil ⁻¹ | | | | | dimensionless |
| 9391 | | 2867 ^{bc} ^a | 217 | 10.7 ^{bc} | 3.63 ^b | 23.1 ^b | 55.3 ^a | 7.29 ^b | 0.37 ^b |
| 9392 | | 2752 ^{bc} | 211 | 11.1 ^a | 3.80 ^{ab} | 25.0 ^{ab} | 53.2 ^{ab} | 6.95 ^b | 0.42 ^{ab} |
| 93B67 | | 2859 ^{bc} | 199 | 11.0 ^{ab} | 3.65 ^b | 26.1 ^a | 52.2 ^b | 7.07 ^b | 0.44 ^a |
| 93B82 | | 3130 ^b | 211 | 10.7 ^{bc} | 3.68 ^b | 25.4 ^{ab} | 53.5 ^{ab} | 6.77 ^b | 0.42 ^{ab} |
| 93M90 | | 3072 ^b | 215 | 10.8 ^{abc} | 3.82 ^{ab} | 23.9 ^{ab} | 53.4 ^{ab} | 8.07 ^a | 0.39 ^{ab} |
| P35T58R | | 3742 ^a | 224 | 10.7 ^{abc} | 4.08 ^a | 23.6 ^{ab} | 54.4 ^{ab} | 7.15 ^b | 0.38 ^{ab} |
| P3981 | | 2433 ^c | 216 | 10.6 ^c | 3.58 ^b | 24.3 ^{ab} | 54.3 ^{ab} | 7.21 ^b | 0.40 ^{ab} |
| | Control | 2748 ^B | 213 | 10.7 | 3.84 ^A | 24.4 | 53.6 | 7.4 | 0.40 |
| | High N | 3211 ^A | 214 | 10.8 | 3.63 ^B | 24.5 | 53.9 | 7.1 | 0.41 |
| Variety | | *** | ns | ** | *** | * | * | *** | * |
| N Treatment | | ** | ns | ns | * | ns | ns | ns | ns |
| Variety x N Treatment | | ns | ns | ns | ns | ns | ns | ns | ns |

^a Different letters represent least significant difference at p<0.05 (Tukey). Lower case letters are used for varieties differences, and capital case for N treatment.

*** p < 0.001; ** p < 0.01; * p < 0.05. ns: not significant

Table 5.3: AIC for all nonlinear models fitted to describe changes in total oil, palmitic, stearic, oleic, linoleic, and linolenic fatty acids during the seed-filling period. AIC values in bold letters were the lowest for each trait and the selected model to describe the content.

| Function | Fixed Effects | Total Oil | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
|---------------------------|---------------------------------------|-------------|-----------|-------------|------------|-------------|-------------|
| Weibull (Equation 4.1) | Variety (V) + N Treatment (N) + V × N | 1689 | 110 | -659 | 886 | 1234 | -279 |
| | V + N | 1675 | 95 | -680 | 877 | 1224 | -292 |
| | N | 1744 | 162 | -600 | 929 | 1297 | -221 |
| | V | 1703 | 118 | -659 | 882 | 1246 | -258 |

Table 5.4: Parameter W_{\max} for the final seed contents for each variety and N treatment of the models fitted for total oil, palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid.

| Variety | N Treatment | Total Oil | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
|-----------------------|-------------|------------------------------|---------------|----------------|----------------|-----------------|----------------|
| mg seed ⁻¹ | | | | | | | |
| 9391 | | 29.0 <i>bcd</i> ^a | 3.1 <i>bc</i> | 1.03 <i>bc</i> | 6.67 <i>b</i> | 16.1 <i>bc</i> | 2.45 <i>a</i> |
| 9392 | | 28.2 <i>cd</i> | 3.2 <i>bc</i> | 1.02 <i>bc</i> | 7.30 <i>b</i> | 14.8 <i>cd</i> | 2.25 <i>ab</i> |
| 93B67 | | 26.8 <i>d</i> | 3.0 <i>c</i> | 0.92 <i>c</i> | 7.37 <i>b</i> | 13.9 <i>d</i> | 2.12 <i>b</i> |
| 93B82 | | 34.6 <i>a</i> | 3.8 <i>a</i> | 1.23 <i>a</i> | 8.90 <i>a</i> | 18.4 <i>a</i> | 2.05 <i>bc</i> |
| 93M90 | | 31.9 <i>ab</i> | 3.5 <i>ab</i> | 1.22 <i>a</i> | 7.79 <i>ab</i> | 17.1 <i>ab</i> | 2.03 <i>bc</i> |
| P35T58R | | 30.4 <i>bc</i> | 3.3 <i>bc</i> | 1.22 <i>a</i> | 7.31 <i>b</i> | 16.6 <i>abc</i> | 1.81 <i>c</i> |
| P3981 | | 31.4 <i>abc</i> | 3.4 <i>b</i> | 1.14 <i>ab</i> | 7.83 <i>ab</i> | 17.0 <i>ab</i> | 1.80 <i>c</i> |
| | Control | 28.0 <i>B</i> | 3.1 <i>B</i> | 1.04 <i>B</i> | 6.98 <i>B</i> | 15.1 <i>B</i> | 1.92 <i>B</i> |
| | High N | 32.6 <i>A</i> | 3.6 <i>A</i> | 1.18 <i>A</i> | 8.21 <i>A</i> | 17.5 <i>A</i> | 2.22 <i>A</i> |

^a Different letters represent least significant difference at $p < 0.05$ (Tukey). Lower case letters are used for varieties differences, and capital case for N treatment.

Chapter 6 - Final remarks

Throughout this dissertation, physiological processes related to nutrient dynamics and seed composition in soybean were described in response to genotype, environmental and management manipulations. The interdependence of seed yield, plant N dynamics and seed composition in the context of crop improvement are relevant to identify physiological constraints. The variations observed in seed yield in response to total plant N uptake (Fig. 2.1) are associated to the N allocated into the seeds (i.e., NHI). Following the theoretical approach from Sinclair (1998), the relationship between NHI and HI was validated with field observations as a curvilinear response mediated by the N concentration in both seed and stover. The implication is that, the N recovery process originated by seed N demand would limit selection for higher HI in soybean. This relationship helps to explain the higher contribution of biomass instead of HI to seed yield increases in soybean in the last decades (Chapter 4) together with the higher plant N uptake (Kumudini, 2002).

The limitations in the use HI is associated with the methodology used in each case and constitutes a source of inconsistency across scientific literature. The alternative analysis in Chapter 4 using allometric relationships between reproductive and vegetative organs complements the interpretation of partitioning indices such as HI. Increases in biomass allocation to reproductive organs were evident when allometric exponents from the log-log relationships between reproductive organs and shoot biomass (Fig. 4.4A) were related to the year of release of the varieties. The fact that HI does not capture increases in biomass partitioning was confirmed using a set of independent studies.

Reproductive allocation also interacts with C and N acquisition. The proposed hypotheses in Chapter 3 introduce this concept through the C cost of fixing atmospheric N for the crop. The

mechanism by which the crop accommodates the cost of BNF were associated with the reduction of seed yield (mediated by HI) and seed oil concentration. The addition of N fertilizer increased productivity enhancing C allocation to the seeds and reducing BNF at the onset of the seed filling period. This analysis may foster new questions in the context of greater N requirements to increase seed yield in soybean and the potential trade-off with C allocation. This remark leads to the mechanistic way by which plant C and N interacts during the seed filling period.

Nitrogen fixation activity tends to decrease when developing seeds attain their maximum growth rate probably due to a reduction in C supply to the nodules (Sinclair and Nogueira, 2018). Carbon supply to the seeds depends on current photosynthesis in leaves whereas N accumulation in seeds depends upon soil and BNF. However, seed N demand cannot be sustained by these sources and vegetative organs undergo N remobilization. The magnitude and efficiency by which this process occurs will depend on the organ and N fertilization (Ortez et al., 2019). Results from Chapters 4 and 5 reinforce the effect of greater N accumulation during vegetative and reproductive stages when the crop grows under high N availability. As discussed in Chapter 5, the greater availability of N increased the length of the effective filling period, delayed canopy senescence and maintain C supply to seed components (e.g., oil and FA). Prospects for future breeding improvement should consider increasing the effect of N remobilization and its interaction with C assimilation. Whether improving efficiency on N remobilization or maintaining N absorption by enhancing BNF are alternatives to boost seed filling (Salon et al., 2001), both of them should not compromise seed nutritional profile.

Finally, the historic component on this dissertation heightens the need for studying the effects of seed yield improvement on seed composition. Seed yield improvement on total oil and FA profile was not biologically relevant in the varieties used in this dissertation. It was not clear

how concentration remained stable against the increases of oil and FA content in seed. This lead to the hypothesis of seed homeostasis in response to greater source availability in these components. The response in protein and carbohydrates concentration merit further investigation in the context of the strong trade-off between protein and seed yield (Chapter 3).

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Appendix A - Figures and Tables Chapter 3

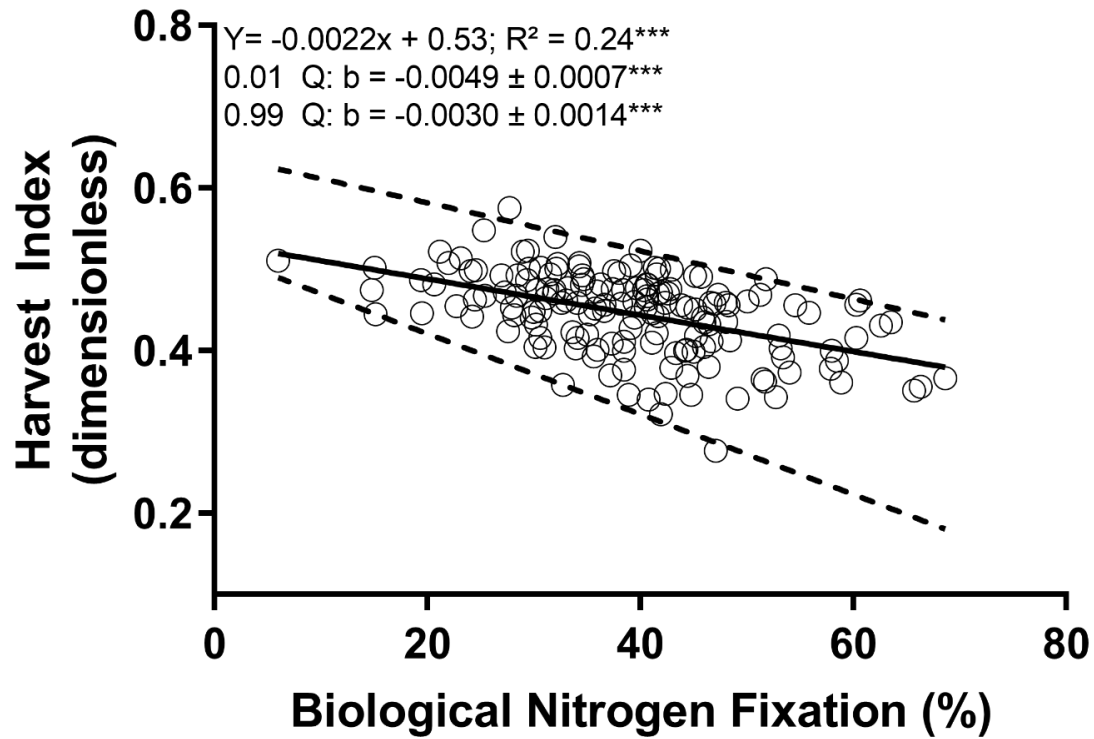


Figure A.1: Relationship between harvest index and biological nitrogen fixation for 20 chickpea (*Cicer arietinum* L.) varieties grown in eight different environments. Solid line represent the least square regression, and dashed lines are regressions for the 0.99 and 0.01 quantiles. Asterisks indicate significance of the coefficient: three asterisks, $P < 0.001$. Original data from Sadras et al. (2016).

Table A.1: Pearson correlation coefficients (r) between variables of the PCA (Fig. 2.4). Traits are maximum RAU during the growing season (RAU_{max}), thermal time for RAU_{max} (t_{max}), maximum rate (MaxRate), relative AUC (AUC), RAU at R6 (RAU_{R6}), residuals from seed yield (Res_Yield), total biomass (Res_Biom), harvest index (Res_HI), oil concentration (Res_Oil), seed protein concentration (Res_Prot), soil organic matter (OM), soil pH, cation exchange capacity (CEC), clay, and sand percentage.

| | RAU _{max} | t _{max} | MaxRate | AUC | RAU _{R6} | Res_Yield | Res_Biom | Res_HI | Res_Oil | Res_Prot | OM | pH | CEC | Clay |
|-------------------|--------------------|------------------|----------|----------|-------------------|-----------|-----------|----------|---------|-----------|----------|----------|-----------|----------|
| t _{max} | -0.04ns | | | | | | | | | | | | | |
| MaxRate | 0.32** | -0.83*** | | | | | | | | | | | | |
| AUC | 0.50*** | 0.46*** | -0.18 * | | | | | | | | | | | |
| RAU _{R6} | 0.76*** | 0.11ns | 0.02 ns | 0.31 ** | | | | | | | | | | |
| Res_Yield | -0.18 * | 0.12ns | -0.15 ns | 0.10ns | -0.29 ** | | | | | | | | | |
| Res_Biom | -0.16ns | 0.07ns | -0.03 ns | -0.13ns | -0.14 ns | 0.60*** | | | | | | | | |
| Res_HI | -0.13ns | -0.04ns | 0.04 ns | 0.18ns | -0.32 ** | 0.40*** | -0.29 ** | | | | | | | |
| Res_Oil | -0.08ns | -0.01ns | -0.10 ns | -0.17ns | -0.21 * | 0.11 ns | 0.12 ns | -0.09 ns | | | | | | |
| Res_Prot | 0.07ns | -0.03ns | 0.14 ns | 0.15ns | 0.01 ns | -0.28 ** | -0.39 *** | -0.04 ns | -0.33ns | | | | | |
| OM | 0.10ns | -0.41 *** | 0.30 ** | -0.32** | 0.22 * | 0.04 ns | -0.05 ns | -0.04 ns | -0.08ns | -0.18 ns | | | | |
| pH | 0.05 ns | -0.39*** | 0.26 * | -0.43*** | 0.16 ns | -0.01 ns | 0.07 ns | -0.16 ns | -0.06ns | -0.35 *** | 0.50 *** | | | |
| CEC | 0.03 ns | -0.27** | 0.08 ns | -0.16ns | 0.05 ns | 0.07 ns | -0.10 ns | -0.17 ns | 0.09ns | -0.25 * | 0.49 *** | 0.46 *** | | |
| Clay | 0.05 ns | -0.29** | 0.13 ns | -0.20* | 0.07 ns | -0.06 ns | -0.12 ns | -0.18 * | 0.09ns | -0.25 * | 0.45 *** | 0.47 *** | 0.97 *** | |
| Sand | 0.21 * | -0.03ns | 0.25 * | -0.21 * | 0.31 ** | -0.16 ns | 0.22 * | -0.05 ns | -0.13ns | -0.11 ns | 0.19 * | 0.13 ns | -0.44 *** | -0.33 ** |

* $p \leq 0.10$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns: $p > 0.10$.

Appendix B - Figures and Tables Chapter 4

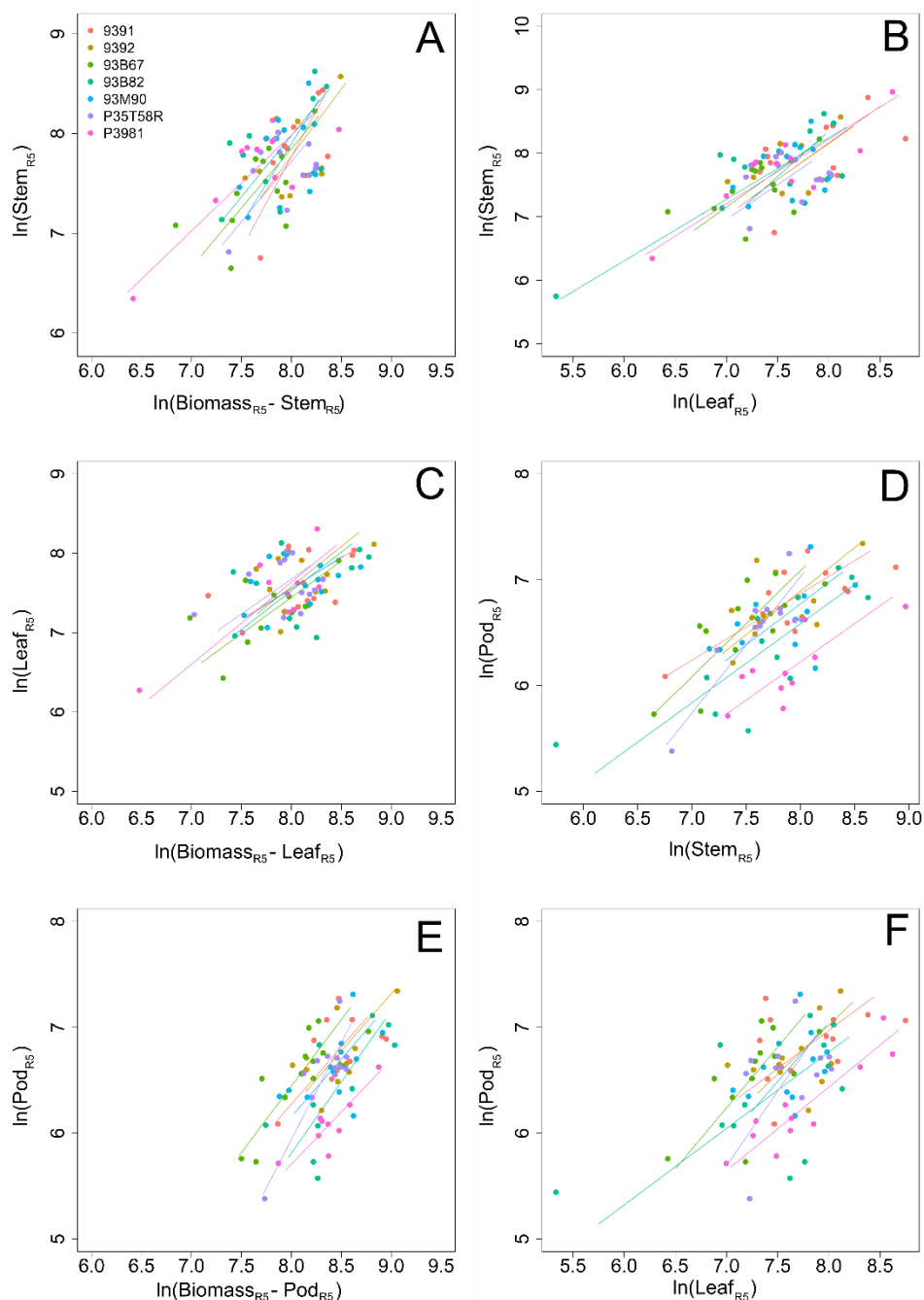


Figure B.1: Allometric (log-log) relationship between stem and shoot biomass (excluding stem; A), stem and leaf biomass (B), leaf and shoot biomass (excluding leaf; C), pod and leaf biomass (D), pod and shoot biomass (excluding pod; E), and pod and stem (F) at the R5 stage. Each circle is the observed value for from each plot.

Table B.1: Intercepts and allometric exponents for allometric (log-log) relationship at R5 for soybean varieties released between 1980 and 2013. Top: relationships between stems and shoot biomass (excluding stem), between leaves and shoot biomass (excluding leaves), and between pods and shoot biomass (excluding pods). Bottom: relationships between stems and leaves, pods and stems, and pods and leaves. Lower and upper 95% confidence intervals are shown for slopes; all slopes were significantly different from zero ($p < 0.001$).

| Year of Release | Variety | log stems vs log (biomass – stems) | | | log leaves vs log (biomass – leaves) | | | log pods vs log (biomass – pods) | | |
|-----------------|---------|------------------------------------|---------------------|----------------------------|--------------------------------------|---------------------|----------------------------|----------------------------------|---------------------|----------------------------|
| | | Intercept | Allometric exponent | Allometric exponent 95% CI | Intercept | Allometric exponent | Allometric exponent 95% CI | Intercept | Allometric exponent | Allometric exponent 95% CI |
| 1980 | P3981 | 0.29 | 0.96 | 0.61 - 1.51 | -0.63 | 1.03 | 0.66 - 1.63 | -2.23 | 0.99 | 0.64 - 1.55 |
| 1987 | 9391 | -7.01 | 1.85 | 0.98 - 3.49 | 1.38 | 0.77 | 0.38 - 1.57 | -2.37 | 1.08 | 0.57 - 2.05 |
| 1992 | 9392 | -3.00 | 1.35 | 0.70 - 2.58 | -0.46 | 1.01 | 0.52 - 1.95 | -2.41 | 1.08 | 0.60 - 1.94 |
| 1997 | 93B82 | -1.86 | 1.23 | 0.67 - 2.27 | -0.27 | 0.97 | 0.50 - 1.89 | -4.96 | 1.35 | 0.81 - 2.24 |
| 2001 | 93B67 | -1.66 | 1.19 | 0.70 - 2.01 | 0.13 | 0.91 | 0.53 - 1.57 | -3.60 | 1.26 | 0.85 - 1.85 |
| 2003 | 93M90 | -4.95 | 1.61 | 0.88 - 2.93 | 1.17 | 0.80 | 0.43 - 1.48 | -2.57 | 1.09 | 0.62 - 1.92 |
| 2013 | P35T58R | -1.93 | 1.21 | 0.66 - 2.21 | 0.65 | 0.88 | 0.46 - 1.67 | -8.69 | 1.83 | 1.24 - 2.69 |

| | | log stems vs log leaves | | | log pods vs log stems | | | log pods vs log leaves | | |
|------|---------|-------------------------|---------------------|----------------------------|-----------------------|---------------------|----------------------------|------------------------|---------------------|----------------------------|
| | | Intercept | Allometric exponent | Allometric exponent 95% CI | Intercept | Allometric exponent | Allometric exponent 95% CI | Intercept | Allometric exponent | Allometric exponent 95% CI |
| 1980 | P3981 | 0.09 | 1.02 | 0.70 - 1.48 | 0.44 | 0.72 | 0.44 - 1.18 | 0.11 | 0.79 | 0.58 - 1.08 |
| 1987 | 9391 | -1.02 | 1.15 | 0.65 - 2.03 | 1.84 | 0.63 | 0.39 - 1.02 | 1.20 | 0.72 | 0.39 - 1.34 |
| 1992 | 9392 | -0.85 | 1.13 | 0.58 - 2.21 | 0.32 | 0.82 | 0.46 - 1.48 | -0.38 | 0.93 | 0.48 - 1.78 |
| 1997 | 93B82 | 0.49 | 0.97 | 0.62 - 1.51 | 0.62 | 0.75 | 0.50 - 1.11 | 0.98 | 0.72 | 0.41 - 1.26 |
| 2001 | 93B67 | -0.89 | 1.15 | 0.65 - 2.04 | -0.93 | 1.00 | 0.65 - 1.55 | -1.82 | 1.15 | 0.70 - 1.88 |
| 2003 | 93M90 | -3.14 | 1.44 | 0.77 - 2.69 | 0.56 | 0.78 | 0.43 - 1.39 | -1.88 | 1.12 | 0.60 - 2.06 |
| 2013 | P35T58R | -0.72 | 1.10 | 0.57 - 2.11 | -3.28 | 1.29 | 0.90 - 1.85 | -4.20 | 1.41 | 0.76 - 2.62 |

Appendix C - Figures and Tables Chapter 5

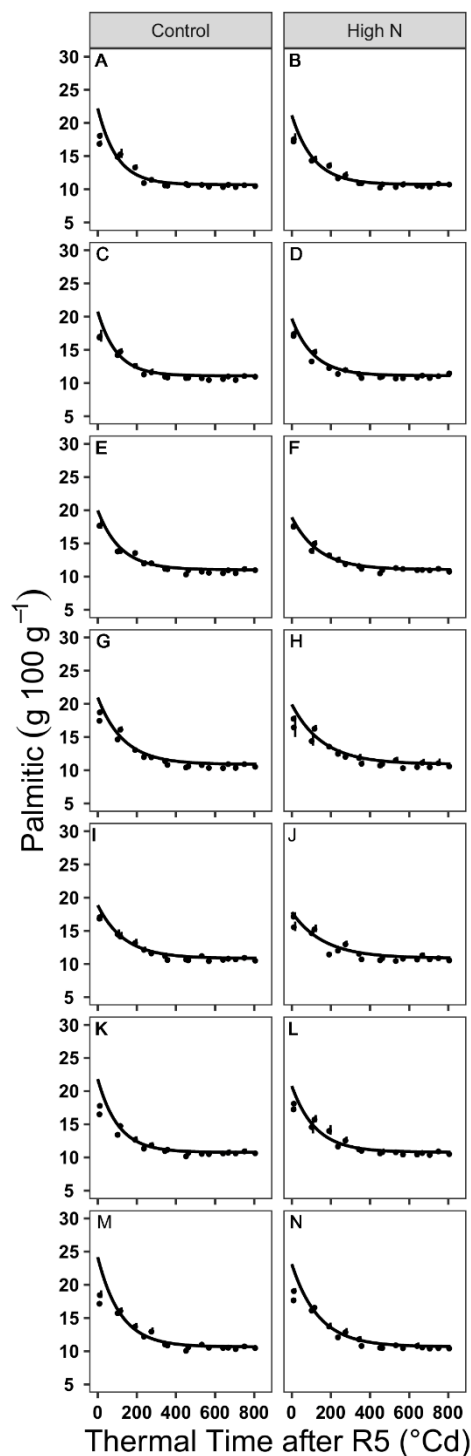


Figure C.1: Changes in palmitic acid concentration during the seed-filling period in each treatment for the 9391 (A-B), 9392 (C-D), 93B67 (E-F), 93B82 (G-H), 93M90 (I-J), P35T58R (K-L), and P3981 (M-N) variety. Vertical bars represent the standard error. Each point represents the average value of three repetitions in each year.

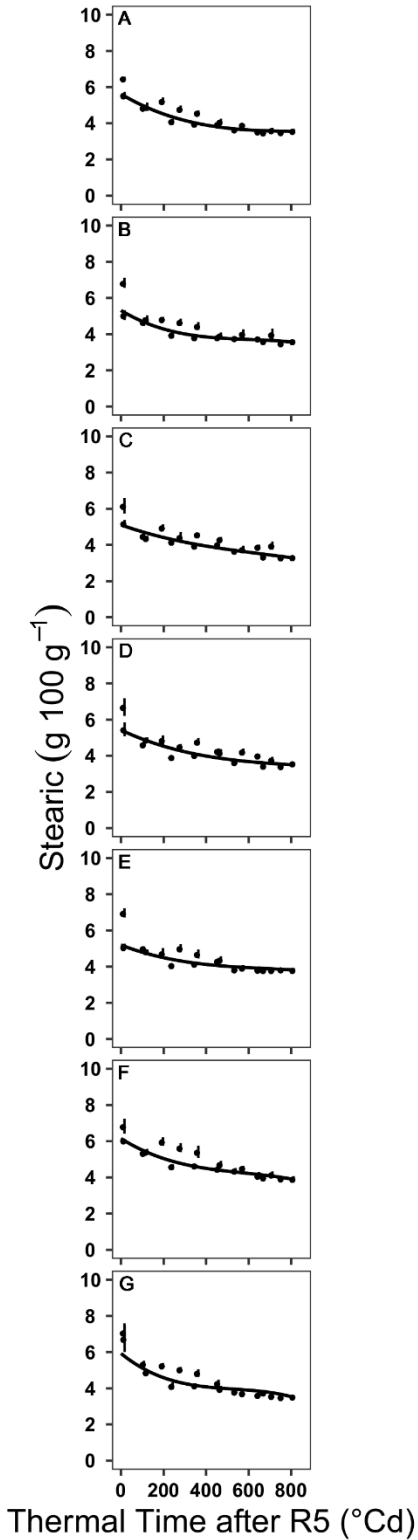


Figure C.2: Changes in stearic acid concentration during the seed-filling period for the 9391 (A), 9392 (B), 93B67 (C), 93B82 (D), 93M90 (E), P35T58R (F), and P3981 (G) variety. Vertical bars represent the standard error. Each point represents the average value of three repetitions in each year and N-treatments.

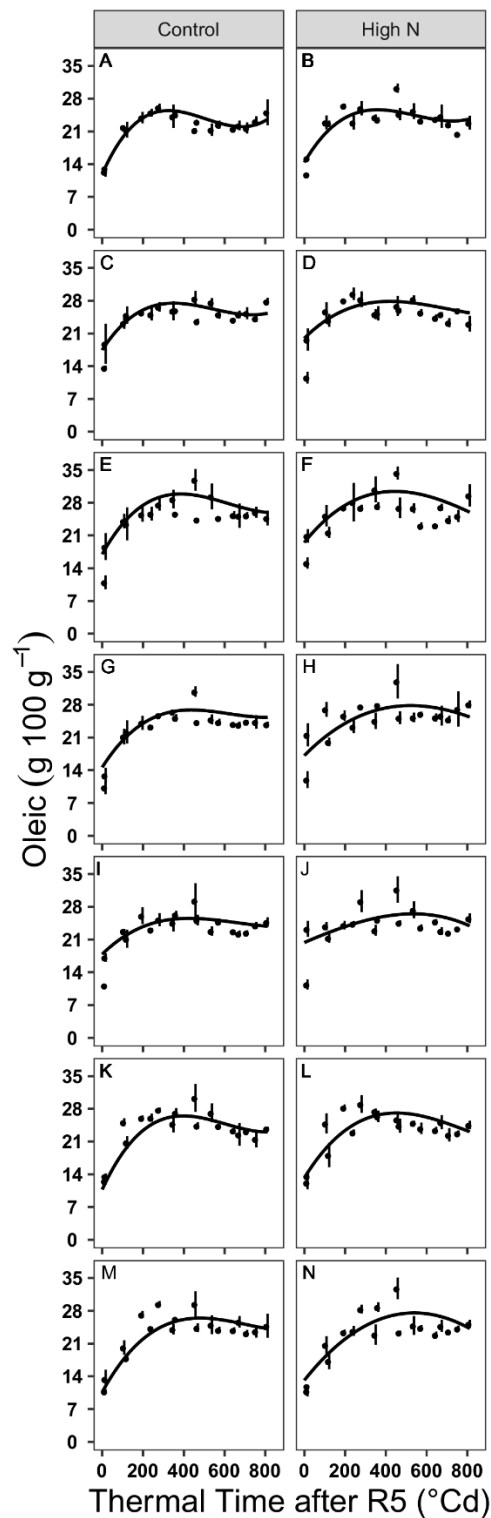


Figure C.3: Changes in oleic acid concentration during the seed-filling period in each treatment for the 9391 (A-B), 9392 (C-D), 93B67 (E-F), 93B82 (G-H), 93M90 (I-J), P35T58R (K-L), and P3981 (M-N) variety. Vertical bars represent the standard error. Each point represents the average value of three repetitions in each year.

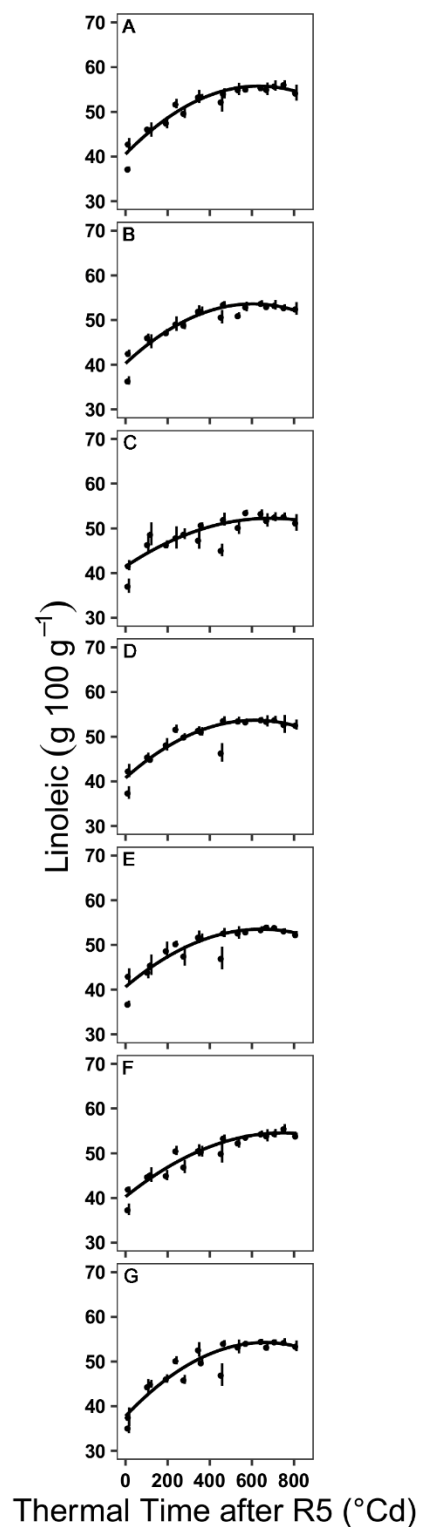


Figure C.4: Changes in linoleic acid concentration during the seed-filling period in each treatment for the 9391 (A), 9392 (B), 93B67 (C), 93B82 (D), 93M90 (E), P35T58R (F), and P3981 (G) variety. Vertical bars represent the standard error. Each point represents the average value of three repetitions in each year and N-treatments.

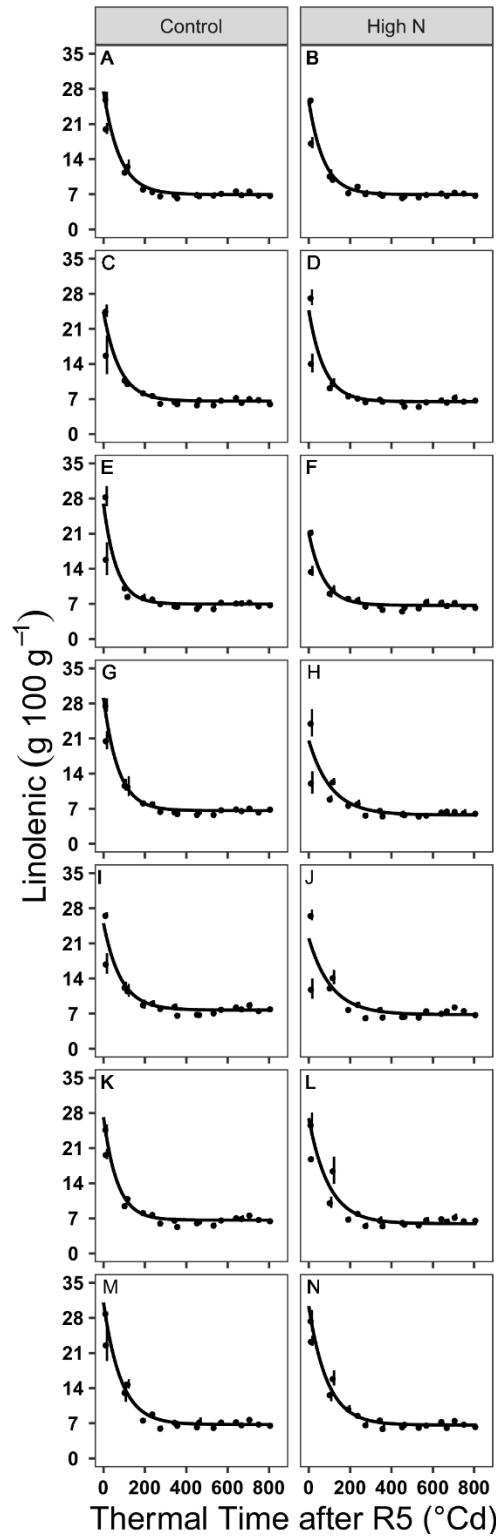


Figure C.5: Changes in linolenic acid concentration during the seed-filling period for the 9391 (A-B), 9392 (C-D), 93B67 (E-F), 93B82 (G-H), 93M90 (I-J), P35T58R (K-L), and P3981 (M-N) variety. Vertical bars represent the standard error. Each point represents the average value of three repetitions in each year.

Table C.1: Parameter for total oil, palmitic, stearic, oleic, linoleic, and linolenic for seed content non-linear models, their standard error, and corresponding fixed effects attached to parameters.

| Seed Component | Curve Parameter | Fixed Effects ^a | Control | | | | | | | High N | | | | | | |
|----------------|------------------|----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| | | | 9391 | 9392 | 93B67 | 93B82 | 93M90 | P35T58R | P3981 | 9391 | 9392 | 93B67 | 93B82 | 93M90 | P35T58R | P3981 |
| Total Oil | W _{max} | V + N | 26.7 ± 1.2 | 25.9 ± 1.2 | 24.6 ± 1.2 | 32.3 ± 1.3 | 29.6 ± 1.3 | 28.1 ± 1.2 | 29.1 ± 1.3 | 31.3 ± 1.2 | 30.4 ± 1.2 | 29.1 ± 1.2 | 36.9 ± 1.3 | 34.2 ± 1.3 | 32.6 ± 1.3 | 33.7 ± 1.3 |
| | Drop | V + N | 26.4 ± 1.2 | 25.5 ± 1.2 | 24.2 ± 1.2 | 31.9 ± 1.3 | 29.2 ± 1.3 | 27.9 ± 1.2 | 29 ± 1.3 | 31 ± 1.2 | 30 ± 1.2 | 28.7 ± 1.2 | 36.4 ± 1.3 | 33.7 ± 1.3 | 32.4 ± 1.3 | 33.5 ± 1.3 |
| | pwr | N | 2.9 ± 0.1 | 2.9 ± 0.1 | 2.9 ± 0.1 | 2.9 ± 0.1 | 2.9 ± 0.1 | 2.9 ± 0.1 | 2.9 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 |
| | lrc | V | -16.9 ± 0.4 | -16.6 ± 0.4 | -16.9 ± 0.4 | -17.1 ± 0.4 | -17.2 ± 0.4 | -17.1 ± 0.4 | -17.3 ± 0.4 | -16.9 ± 0.4 | -16.6 ± 0.4 | -16.9 ± 0.4 | -17.1 ± 0.4 | -17.2 ± 0.4 | -17.1 ± 0.4 | -17.3 ± 0.4 |
| Palmitic | W _{max} | V + N | 2.9 ± 0.1 | 2.9 ± 0.1 | 2.7 ± 0.1 | 3.6 ± 0.1 | 3.3 ± 0.1 | 3.1 ± 0.1 | 3.1 ± 0.1 | 3.4 ± 0.1 | 3.4 ± 0.1 | 3.2 ± 0.1 | 4.1 ± 0.1 | 3.8 ± 0.1 | 3.6 ± 0.1 | 3.6 ± 0.1 |
| | Drop | V + N | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.7 ± 0.1 | 3.5 ± 0.1 | 3.2 ± 0.1 | 3 ± 0.1 | 3.1 ± 0.1 | 3.4 ± 0.1 | 3.3 ± 0.1 | 3.2 ± 0.1 | 4 ± 0.1 | 3.7 ± 0.1 | 3.5 ± 0.1 | 3.6 ± 0.1 |
| | pwr | - | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 |
| | lrc | V + N | -15.5 ± 0.4 | -15.2 ± 0.4 | -15.4 ± 0.4 | -15.6 ± 0.4 | -15.8 ± 0.4 | -15.6 ± 0.4 | -15.8 ± 0.4 | -15.8 ± 0.4 | -15.5 ± 0.4 | -15.7 ± 0.4 | -15.9 ± 0.4 | -16.1 ± 0.4 | -15.9 ± 0.4 | -16.1 ± 0.4 |
| Stearic | W _{max} | V + N | 1 ± 0.04 | 1 ± 0.04 | 0.9 ± 0.04 | 1.2 ± 0.04 | 1.2 ± 0.04 | 1.2 ± 0.04 | 1.1 ± 0.04 | 1.1 ± 0.04 | 1.1 ± 0.04 | 1 ± 0.04 | 1.3 ± 0.04 | 1.3 ± 0.04 | 1.3 ± 0.04 | 1.2 ± 0.04 |
| | Drop | V + N | 0.9 ± 0.04 | 0.9 ± 0.04 | 0.8 ± 0.04 | 1.1 ± 0.04 | 1.1 ± 0.04 | 1.1 ± 0.04 | 1.1 ± 0.04 | 1.1 ± 0.04 | 1.1 ± 0.04 | 1 ± 0.04 | 1.3 ± 0.04 | 1.3 ± 0.04 | 1.3 ± 0.04 | 1.2 ± 0.04 |
| | pwr | - | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 | 3 ± 0.1 |
| | lrc | V + N | -17 ± 0.5 | -16.8 ± 0.5 | -16.9 ± 0.5 | -17.2 ± 0.5 | -17.4 ± 0.5 | -17.2 ± 0.5 | -17.4 ± 0.5 | -17.3 ± 0.5 | -17.1 ± 0.5 | -17.2 ± 0.5 | -17.5 ± 0.5 | -17.7 ± 0.5 | -17.5 ± 0.5 | -17.7 ± 0.5 |
| Oleic | W _{max} | V + N | 6.1 ± 0.4 | 6.7 ± 0.4 | 6.8 ± 0.4 | 8.3 ± 0.4 | 7.2 ± 0.4 | 6.7 ± 0.4 | 7.2 ± 0.4 | 7.3 ± 0.4 | 7.9 ± 0.4 | 8 ± 0.4 | 9.5 ± 0.4 | 8.4 ± 0.4 | 7.9 ± 0.4 | 8.4 ± 0.4 |
| | Drop | V + N | 6 ± 0.4 | 6.6 ± 0.4 | 6.7 ± 0.4 | 8.2 ± 0.4 | 7.1 ± 0.4 | 6.7 ± 0.4 | 7.2 ± 0.4 | 7.2 ± 0.4 | 7.8 ± 0.4 | 7.9 ± 0.4 | 9.4 ± 0.4 | 8.3 ± 0.4 | 7.9 ± 0.4 | 8.4 ± 0.4 |
| | pwr | - | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.2 ± 0.1 |
| | lrc | V + N | -18.5 ± 0.6 | -18.3 ± 0.6 | -18.5 ± 0.6 | -18.8 ± 0.6 | -19 ± 0.6 | -18.7 ± 0.6 | -19.1 ± 0.6 | -18.8 ± 0.6 | -18.6 ± 0.6 | -18.8 ± 0.6 | -19.1 ± 0.6 | -19.3 ± 0.6 | -19 ± 0.6 | -19.4 ± 0.6 |
| Linoleic | W _{max} | V + N | 14.9 ± 0.5 | 13.5 ± 0.5 | 12.7 ± 0.5 | 17.2 ± 0.6 | 15.9 ± 0.6 | 15.4 ± 0.6 | 15.8 ± 0.6 | 17.3 ± 0.6 | 16 ± 0.5 | 15.1 ± 0.5 | 19.7 ± 0.6 | 18.3 ± 0.6 | 17.8 ± 0.6 | 18.2 ± 0.7 |
| | Drop | V + N | 14.8 ± 0.5 | 13.3 ± 0.5 | 12.5 ± 0.5 | 17 ± 0.6 | 15.7 ± 0.6 | 15.3 ± 0.6 | 15.8 ± 0.6 | 17.2 ± 0.6 | 15.8 ± 0.5 | 14.9 ± 0.5 | 19.5 ± 0.6 | 18.1 ± 0.6 | 17.8 ± 0.6 | 18.2 ± 0.7 |
| | pwr | V + N | 2.9 ± 0.1 | 2.9 ± 0.1 | 2.9 ± 0.1 | 2.9 ± 0.1 | 2.8 ± 0.1 | 2.9 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.9 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 |
| | lrc | - | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 | -16.9 ± 0.4 |
| Linolenic | W _{max} | V + N | 1.9 ± 0.1 | 1.7 ± 0.1 | 1.6 ± 0.1 | 2.1 ± 0.1 | 2.3 ± 0.1 | 1.9 ± 0.1 | 2 ± 0.1 | 2.2 ± 0.1 | 2 ± 0.1 | 1.9 ± 0.1 | 2.4 ± 0.1 | 2.6 ± 0.1 | 2.2 ± 0.1 | 2.3 ± 0.1 |
| | Drop | V + N | 1.8 ± 0.1 | 1.6 ± 0.1 | 1.6 ± 0.1 | 2 ± 0.1 | 2.2 ± 0.1 | 1.9 ± 0.1 | 1.9 ± 0.1 | 2.1 ± 0.1 | 1.9 ± 0.1 | 1.9 ± 0.1 | 2.3 ± 0.1 | 2.5 ± 0.1 | 2.2 ± 0.1 | 2.2 ± 0.1 |
| | pwr | V + N | 2.5 ± 0.1 | 2.5 ± 0.1 | 2.5 ± 0.1 | 2.4 ± 0.1 | 2.4 ± 0.1 | 2.4 ± 0.1 | 2.4 ± 0.1 | 2.4 ± 0.1 | 2.5 ± 0.1 | 2.4 ± 0.1 | 2.4 ± 0.1 | 2.4 ± 0.1 | 2.4 ± 0.1 | 2.4 ± 0.1 |
| | lrc | - | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 | -14.4 ± 0.3 |

^a V, Variety; N, N Treatment

Table C.2: AIC for all nonlinear models fitted in Supplementary Figure 4.1-5 to describe changes in palmitic, stearic, oleic, linoleic, and linolenic fatty acids during the seed-filling period. AIC values in bold letters were the lowest for each trait and the selected model to describe the content.

| Fixed Effects | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
|---------------------------------------|------------|------------|-------------|-------------|-------------|
| Variety (V) + N Treatment (N) + V × N | 754 | 135 | 2058 | 3770 | 2849 |
| V + N | 727 | 121 | 2049 | 3752 | 2879 |
| N | 754 | 285 | 2079 | 3798 | 3051 |
| V | 730 | 120 | 2049 | 3751 | 2888 |

Table C.3: Parameter for palmitic, oleic, and linolenic (Figures 4.1, 4.3, 4.5) for seed concentration non-linear models and their standard error.

| N Treatment | Variety | Palmitic | | | Oleic | | | | Linolenic | | |
|-------------|---------|------------|------------|---------------|------------|-------------|-------------------|--------------------------|-----------|-----------|---------------|
| | | $Y0$ | NS | k | a | b | c | d | $Y0$ | NS | k |
| High-N | 9391 | 21.2 ± 1.4 | 10.7 ± 0.1 | 0.009 ± 0.001 | 14.4 ± 1.4 | 0.07 ± 0.02 | -0.0002 ± 0.0001 | 0.0000001 ± 0.00000004 | 26 ± 5 | 6.9 ± 0.3 | 0.014 ± 0.004 |
| | 9392 | 19.7 ± 1.2 | 11.1 ± 0.1 | 0.009 ± 0.001 | 20 ± 1.5 | 0.04 ± 0.02 | -0.0001 ± 0.0001 | 0.00000004 ± 0.00000004 | 25 ± 5 | 6.5 ± 0.3 | 0.013 ± 0.004 |
| | 93B67 | 18.9 ± 1.1 | 11.1 ± 0.1 | 0.007 ± 0.001 | 19.5 ± 1.5 | 0.05 ± 0.02 | -0.0001 ± 0.0001 | 0.00000003 ± 0.00000004 | 21 ± 5 | 6.7 ± 0.3 | 0.014 ± 0.004 |
| | 93B82 | 19.9 ± 1.1 | 10.9 ± 0.1 | 0.006 ± 0.001 | 17.1 ± 1.5 | 0.04 ± 0.02 | -0.0001 ± 0.0001 | 0.00000001 ± 0.00000004 | 21 ± 5 | 5.8 ± 0.3 | 0.009 ± 0.003 |
| | 93M90 | 17.8 ± 0.9 | 10.9 ± 0.1 | 0.006 ± 0.001 | 20.4 ± 1.5 | 0.02 ± 0.02 | -0.00001 ± 0.0001 | -0.00000001 ± 0.00000004 | 22 ± 5 | 6.8 ± 0.3 | 0.009 ± 0.003 |
| | P35T58R | 20.8 ± 1.3 | 10.8 ± 0.1 | 0.008 ± 0.001 | 13.3 ± 1.4 | 0.07 ± 0.02 | -0.0001 ± 0.0001 | 0.00000005 ± 0.00000004 | 27 ± 5 | 6 ± 0.3 | 0.01 ± 0.003 |
| | P3981 | 23.1 ± 1.8 | 10.7 ± 0.1 | 0.007 ± 0.001 | 13.2 ± 1.6 | 0.05 ± 0.02 | -0.0001 ± 0.0001 | 0.00000001 ± 0.00000004 | 30 ± 5 | 6.6 ± 0.3 | 0.011 ± 0.003 |
| Control | 9391 | 22.2 ± 1.4 | 10.7 ± 0.1 | 0.01 ± 0.001 | 11.9 ± 1.4 | 0.1 ± 0.02 | -0.0002 ± 0.0001 | 0.00000015 ± 0.00000004 | 28 ± 5 | 6.9 ± 0.3 | 0.013 ± 0.003 |
| | 9392 | 20.8 ± 1.2 | 11.1 ± 0.1 | 0.01 ± 0.001 | 17.5 ± 1.5 | 0.07 ± 0.02 | -0.0001 ± 0.0001 | 0.00000009 ± 0.00000004 | 24 ± 5 | 6.6 ± 0.3 | 0.013 ± 0.004 |
| | 93B67 | 20 ± 1.1 | 11 ± 0.1 | 0.009 ± 0.001 | 17 ± 1.5 | 0.08 ± 0.02 | -0.0001 ± 0.0001 | 0.00000008 ± 0.00000004 | 27 ± 5 | 7 ± 0.3 | 0.016 ± 0.004 |
| | 93B82 | 21 ± 1.2 | 10.9 ± 0.1 | 0.008 ± 0.001 | 14.6 ± 1.4 | 0.07 ± 0.02 | -0.0001 ± 0.0001 | 0.00000007 ± 0.00000004 | 29 ± 5 | 6.6 ± 0.3 | 0.014 ± 0.003 |
| | 93M90 | 18.9 ± 0.9 | 10.8 ± 0.1 | 0.007 ± 0.001 | 17.9 ± 1.5 | 0.04 ± 0.02 | -0.0001 ± 0.0001 | 0.00000004 ± 0.00000004 | 25 ± 5 | 7.7 ± 0.3 | 0.012 ± 0.003 |
| | P35T58R | 21.8 ± 1.3 | 10.8 ± 0.1 | 0.01 ± 0.001 | 10.8 ± 1.3 | 0.09 ± 0.02 | -0.0002 ± 0.0001 | 0.00000001 ± 0.00000004 | 27 ± 5 | 6.7 ± 0.3 | 0.015 ± 0.004 |
| | P3981 | 24.2 ± 1.8 | 10.6 ± 0.1 | 0.009 ± 0.001 | 10.7 ± 1.5 | 0.08 ± 0.02 | -0.0001 ± 0.0001 | 0.00000006 ± 0.00000004 | 31 ± 5 | 6.7 ± 0.3 | 0.012 ± 0.003 |

Table C.4: Parameter for stearic and linoleic (Figures 4.2 and 4.4) for seed concentration non-linear models and their standard error.

| Variety | Stearic | | | | Linoleic | | |
|---------|---------------|------------------|--------------------|----------------------|----------------|-----------------|------------------------|
| | <i>a</i> | <i>b</i> | <i>c</i> | <i>d</i> | <i>a</i> | <i>b</i> | <i>c</i> |
| P3981 | 5.6 ± 0.2 | 0.2 ± -0.01 | -0.01 ± 0.002 | 0.002 ± 0.00001 | 40.5 ± 1.6 | 0.05 ± 0.01 | -0.00004 ± 0.00001 |
| 9391 | 5.3 ± 0.2 | 0.2 ± -0.01 | -0.01 ± 0.002 | 0.002 ± 0.00001 | 40.3 ± 1.6 | 0.04 ± 0.01 | -0.00004 ± 0.00001 |
| 9392 | 5.1 ± 0.2 | 0.2 ± -0.004 | -0.004 ± 0.001 | 0.001 ± 0.000004 | 41.5 ± 1.6 | 0.03 ± 0.01 | -0.00002 ± 0.00001 |
| 93B67 | 5.4 ± 0.2 | 0.2 ± -0.01 | -0.01 ± 0.002 | 0.002 ± 0.00001 | 40.8 ± 1.6 | 0.04 ± 0.01 | -0.00003 ± 0.00001 |
| 93B82 | 5.2 ± 0.2 | 0.2 ± -0.005 | -0.005 ± 0.002 | 0.002 ± 0.00001 | 40.6 ± 1.6 | 0.04 ± 0.01 | -0.00003 ± 0.00001 |
| 93M90 | 6.2 ± 0.3 | 0.3 ± -0.01 | -0.01 ± 0.002 | 0.002 ± 0.00001 | 40.3 ± 1.6 | 0.04 ± 0.01 | -0.00003 ± 0.00001 |
| P35T58R | 5.9 ± 0.3 | 0.3 ± -0.01 | -0.01 ± 0.002 | 0.002 ± 0.00002 | 37.8 ± 1.6 | 0.05 ± 0.01 | -0.00004 ± 0.00001 |

Table C.5: Average \pm standard error of total oil, palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid content for each variety and N treatment. Each observation is the average of all repetitions from the two years of experiment.

| Treatment | Variety | Total oil | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
|-----------------------|---------|----------------|---------------|---------------|---------------|----------------|---------------|
| mg seed ⁻¹ | | | | | | | |
| Control | 9391 | 26.2 \pm 1.5 | 2.8 \pm 0.2 | 1.0 \pm 0.1 | 6.2 \pm 0.6 | 14.4 \pm 0.9 | 1.9 \pm 0.1 |
| | 9392 | 22.6 \pm 1.7 | 2.5 \pm 0.2 | 0.9 \pm 0.1 | 6.0 \pm 0.4 | 11.7 \pm 1.0 | 1.5 \pm 0.2 |
| | 93B67 | 26.9 \pm 1.4 | 2.9 \pm 0.1 | 1.0 \pm 0.1 | 6.8 \pm 0.6 | 14.2 \pm 0.6 | 1.9 \pm 0.1 |
| | 93B82 | 29.4 \pm 1.5 | 3.1 \pm 0.2 | 1.1 \pm 0.1 | 7.1 \pm 0.4 | 16.0 \pm 0.8 | 2.1 \pm 0.1 |
| | 93M90 | 28.6 \pm 1.3 | 3.1 \pm 0.2 | 1.1 \pm 0.1 | 6.8 \pm 0.3 | 15.3 \pm 0.8 | 2.4 \pm 0.1 |
| | P35T58R | 28.1 \pm 2.6 | 3.0 \pm 0.3 | 1.2 \pm 0.1 | 6.6 \pm 0.6 | 15.2 \pm 1.5 | 2.0 \pm 0.2 |
| | P3981 | 29.7 \pm 2.2 | 3.1 \pm 0.2 | 1.1 \pm 0.1 | 7.2 \pm 0.8 | 16.1 \pm 1.1 | 2.2 \pm 0.2 |
| High N | 9391 | 32.2 \pm 2.2 | 3.5 \pm 0.3 | 1.1 \pm 0.1 | 7.4 \pm 0.7 | 17.9 \pm 1.0 | 2.3 \pm 0.2 |
| | 9392 | 29.7 \pm 1.1 | 3.3 \pm 0.1 | 1.1 \pm 0.1 | 7.0 \pm 0.5 | 16.2 \pm 0.5 | 2.1 \pm 0.1 |
| | 93B67 | 29.6 \pm 2.0 | 3.3 \pm 0.2 | 1.0 \pm 0.1 | 8.0 \pm 0.6 | 15.3 \pm 1.2 | 2.1 \pm 0.2 |
| | 93B82 | 36.5 \pm 1.2 | 3.9 \pm 0.1 | 1.4 \pm 0.1 | 9.7 \pm 0.5 | 19.2 \pm 0.5 | 2.3 \pm 0.1 |
| | 93M90 | 34.2 \pm 0.6 | 3.7 \pm 0.1 | 1.3 \pm 0.1 | 8.2 \pm 0.3 | 18.3 \pm 0.4 | 2.6 \pm 0.1 |
| | P35T58R | 31.2 \pm 1.1 | 3.3 \pm 0.1 | 1.2 \pm 0.1 | 7.3 \pm 0.4 | 17.1 \pm 0.7 | 2.2 \pm 0.1 |
| | P3981 | 31.4 \pm 1.4 | 3.3 \pm 0.2 | 1.1 \pm 0.1 | 7.7 \pm 0.4 | 17.0 \pm 0.7 | 2.2 \pm 0.1 |