Interrelationships between Soybean Seed Quality Characteristics

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Chapter 1 – Literature Review

Physiology of Soybean Yield Production

Production of soybean

Soybean is an important crop with 2015/2016 global production of 325 million metric tons supplying 70% of world protein meal and 30% of vegetable oil (USDA-NASS, 2016). Protein and oil typically make up 535 g kg^{-1} of soybean yield (13% moisture basis) with 340 g kg^{-1} protein and 195 g kg^{-1} oil (Hurburgh et al., 1987). Because of the high market demand for protein and oil, total seed yield improvement in soybean should also have a neutral or positive effect on the seed concentration of protein and oil.

Protein and oil physiology

Protein, oil, and carbohydrates are the three main macromolecule groups of interest in soybean yield. Penning de Vries (1974) prescribed each group a biosynthetic production value such that one unit of glucose, oxygen, and mineral nutrient is required to produce 0.330, 0.404, and 0.826 units of oil, protein, and carbohydrate, respectively. The production value of macromolecules was then combined with seed composition values to characterize the seed yield of major crops in terms of biomass productivity (g seed/ g glucose) and N requirement (mg N/ g glucose) (Sinclair and de Wit, 1975). Soybean had the highest N requirement (29 mg N/ g glucose), similar to the N requirement of pulses. Soybean had a low seed biomass productivity (0.50 g seed/ g glucose) which was similar to oilseeds. Overall, the results demonstrate that soybean has unique seed composition which requires both C and N in abundance (Sinclair and de Wit, 1975). The high energy demands of both protein and oil help explain their negative relationship (Rotundo et al.,

2016). Additionally, the energy demands on N procurement for protein help explain the negative relationship between protein and yield (Krober and Cartter, 1962; Hymowitz et al., 1972; Wilcox and Zhang, 1997; Rotundo et al., 2009).

Mechanisms of carbon and nitrogen supply

As discussed, the soybean seed has a large requirement for C and N. The developing seed is physically separated from the vegetative plant structures by the embryo sac, leaving it entirely dependent on C and N compounds supplied by the mother plant through the two large vascular bundles (Thorne, 1981). The beginning of pod filling (R5, Fehr et al., 1971) coincides with maximum vegetative dry weight and leaf area index for indeterminate-type soybean (Kumudini et al., 2001). Pod filling also marks the maximum photosynthetic output as leaf area decreases steadily to zero at maturity (Kumudini et al., 2001; Kumudini, 2002). The majority of C supplied to the developing embryos comes from phloem-delivered sugar, primarily in the form of sucrose (Layzell and Larue, 1982; Rainbird et al., 1984). The amide amino acids glutamine and asparagine account for three-quarters of the N supplied to the embryo with additional contributions to pod N status from ureides, ammonia, and other amino acids (Layzell and Larue, 1982; Ohyama and Kawai, 1983; Rainbird et al., 1984). Soybeans accumulate N through both nitrate uptake and dinitrogen fixation, with primary molecular transportation being nitrate and ureides, respectively (McClure and Israel, 1979; Ohyama and Kumazawa, 1979). Nitrogen fixation increases rapidly at the beginning of soybean reproductive stages (R1). Fixation supplies 65% of total N assimilated during pod fill, a

majority of N found in the seeds, and half of total season assimilated N (Zapata et al., 1987). Nitrogen from biological N fixation in the nodules can be directly incorporated into the developing seed or incorporated into vegetative tissues. Absorbed nitrate is only incorporated into vegetative proteins before possible remobilization, supporting the primacy of N supplied from fixation on seed development (Ohyama, 1983). Remobilization of the N reserves in the leaves, and to a lesser extent the stem, petioles, and pod walls, also supplies N to the developing embryos (Zeiher et al., 1982; Zhao et al., 2014). This supply is significant as C from current photosynthesis carries N supplying between 12 and 48 percent of seed N through the phloem (Koch and Schrader, 1984). Research in pigeon pea has shown similar results where 35-47% of seed N is supplied by remobilization, depending on the cultivar (Sanetra et al., 1998). Because the primary source of remobilized N is vegetative protein, most remobilized N is transported in the form of free amino acids or small peptides (Havé et al., 2016). The makeup of remobilized N will then likely be influenced by the amino acid composition of leaf proteins. In addition to N, at least 40% of leaf supplies of C, K, P, S and metals were mobilized in a careful study of *Arabidopsis thaliana* leaf senescence (Himelblau and Amasino, 2001). This underscores the importance of the vegetative remobilization process to supply the developing seed with the complex elemental resources it needs for yield formation.

Whole plant physiology determines yield and senescence

It was originally hypothesized that the N demand of the embryos accelerated plant senescence by repurposing N present in photosynthetic enzymes. Therefore, creating a larger vegetative reserve and/or increasing N procurement from uptake and fixation would slow senescence and increase yield (Sinclair and de Wit, 1975, 1976). The overall picture has proven to be more complex and involves plant-level relationships. Soybean plants freed of reproductive tissues, and subsequent N demand, began leaf senescence at the same time as controls (Crafts-Brandner and Egli, 1987). Also, shade removal at beginning of seed fill increased N transport to the seed but did not accelerate leaf senescence (Hayati et al., 1995). Taken together this would suggest that senescence is the result of physiological processes within the entire plant and not a simple function of seed demand. Additionally, the amount of seed N from redistribution was not related to seed yield or length of seed filling across a group of cultivars (Zeiher et al., 1982). High protein lines yielded more seed N per area but had similar vegetative N reserves and leaf senescence patterns as normal protein cultivars; the extra N in the high protein lines likely originating from N accrual during seed filling (Egli and Bruening, 2007). Overall, vegetative N supplies are not well correlated with plant yields and vary widely across environments. Gains in protein yields have been made due to whole plant relationships that favor concurrent N accumulation from nitrate uptake and dinitrogen fixation during seed filling (Zeiher et al., 1982; Shibles and Sundberg, 1998; Egli and Bruening, 2007; Zhao et al., 2014). However, remobilized N may be important in short-season environments with variable conditions during seed fill as evidenced by the positive

correlation (r = 0.45) between R5.5 canopy reserves and yield across ten Minnesota environments, with stronger correlations observed at the lowest-yielding environments (Naeve et al., 2008).

Soybean Seed Quality Interrelationships

Effect of temperature and precipitation

Temperature and precipitation during seed filling correlate with changes in oil, protein, and protein plus oil as concentration of yield (Howell and Cartter, 1959; Piper and Boote, 1999; Naeve and Huerd, 2008; Rotundo and Westgate, 2009). Oil concentration increases rapidly with temperature as does the sum of protein and oil concentrations. The slope of the interaction increases with latitude suggesting greater dependence between temperature and quality in northern environments (Piper and Boote, 1999). Precipitation also has a positive relationship with protein and oil concentrations, with protein being reduced less than oil under water stress conditions (Maestri et al., 1998; Rotundo and Westgate, 2009).

Regional trends

Surveys both within the soybean-growing regions of the United States and between the U.S. and other soybean-producing countries have revealed differences in protein and oil composition (Hurburgh et al., 1990; Grieshop et al., 2003; Thakur and Hurburgh, 2007; Rotundo et al., 2016). In the U.S., soybeans grown in the north-west Corn Belt states of Wisconsin, Minnesota, Nebraska, South Dakota and North Dakota sometimes

have one or two percentage points less protein than soybeans from other production areas (Breene et al., 1988; Hurburgh et al., 1990; Hurburgh, 1994; Thakur and Hurburgh, 2007). These regional differences result in lower demand from buyers. This discrimination is partially facilitated by a distinct geographic separation of shipping routes; most north-west Corn Belt soybeans travel to the Pacific Northwest railway to ports on the west coast (PNW), and other regions use the Mississippi corridor to the Gulf of Mexico ports (NOLA). Price reduction due to lower protein concentrations has been an issue in PNW railroad linked areas of the north-west Corn Belt where seed protein levels are around 340 g kg^{-1} or lower (Hurburgh, 1994; Miller-Garvin and Naeve, 2015). The quality discount for PNW originated soybeans is estimated at forty cents a bushel (William W. Wilson, personal communication, 2016). Processors prefer soybeans with more than 340 g kg^{-1} protein because below this protein concentration the soybeans require partial or complete dehulling to meet 440 g kg^{-1} protein minimum requirements for processed soybean meal. Dehulling results in decreased meal yield, increased manufacturing costs, and a less desirable byproduct (Hurburgh et al., 1990; Hurburgh, 1994).

Amino acids

It has long been reported that amino acids, the fundamental units of protein, account for the value of a protein source in terms of animal and human growth (Osborne and Mendel, 1914). There are eighteen amino acids commonly reported in protein analysis. Those amino acids that are required in the diet of monogastric animals for growth and

development are deemed essential while those that can be produced from other amino acids are deemed non-essential (Boisen et al., 2000). Since the essential amino acids cannot be synthesized, if they are present in rations at lower concentrations than required the result is sub-optimal growth. Therefore, there is an obvious benefit to monitoring performance and growth limiting amino acids. Determining limiting amino acids requires context of intended use. Required amino acid ratios vary both between animal species and within species by factors such as breed, sex, and developmental stage (Berry et al., 1962; Fernandez et al., 1994; Boisen et al., 2000). Across the range of demand, soybean protein has five limiting amino acids: methionine, cysteine, threonine, lysine, and tryptophan (Thakur and Hurburgh, 2007). Increasing these five limiting amino acids in soybean protein would reduce the need for synthetic amino acid supplementation. Balanced amino acid rations result in less excess amino acid deamination and excretion of N as waste, a benefit to animal growth and the environment (Berry et al., 1962).

Whole soybeans as a commodity are still largely valued by a measurement of crude protein alone even though concentration of amino acids controls growth. This is based on the assumption that soybean protein and amino acid balance have a static proportional relationship. Recent research has attested to variation in soybean protein composition. In an analysis of soybean meal samples from the three main soybean-producing countries (U.S., Brazil and Argentina) as well as China and India, there was a negative relationship between protein and the balance of the five limiting amino acids (Thakur and Hurburgh, 2007). The average amino acid balance of the five limiting amino acids in the U.S. meal was statistically greater than that in meal from Argentina or India, indicating greater

nutritional value of the protein in the U.S. meal samples (Thakur and Hurburgh, 2007). This was supported by Wang and coworkers who showed that pigs fed U.S. origin soybean meal had greater average daily gain and feed efficiency than pigs fed soybean meal from Brazil or India (Wang et al., 2011).

Changes in the amino acid balance also have a genetic component. Zarkadas and coworkers found differences between 14 public and private cultivars in amino acid relative amounts [(g amino acid/ g protein)*100], with ranges in the seven most abundant amino acids differing statistically between some cultivars: glutamic acid (16.9-19.3%), aspartic acid (10.6-11.5%), valine (7.5-8.1%), leucine (7.2-8.1%), arginine (6.2-7.7%), lysine (6.0-6.7%), phenylalanine (5.1-5.5%), total (59.5-63.7%) (Zarkadas et al., 2007). Differences in the ranges of the other limiting amino acids: methionine (1.6-2.2%), cysteine (1.9-2.4%), threonine (4.8-5.2%), and tryptophan (1.2-1.5%) were also found (Zarkadas et al., 2007). Changes in the relative abundance of amino acids could be related to seed protein concentration. A characterization of a decade of U.S. soybean sample analysis showed that the amino acid balance of all five limiting amino acids decreased with increased protein concentration, and the balance was made up by increased relative abundance of glutamic acid and arginine within the protein (Medic et al., 2014). Amino acid analysis of four high-protein lines revealed that they were all enriched in glutamic acid and arginine compared to two check varieties, and some showed a decreased abundance of threonine, methionine, and lysine (Serretti et al., 1994). It was also noted that lines with higher protein concentration had the lowest S amino acid (methionine and cysteine) concentration on a percent of protein basis (Zarkadas et al.,

2007). Both genetic and environmental influences can affect the balance of amino acids, with a negative relationship between the abundance of the limiting amino acids and the seed protein concentration.

Source-sink treatments

Studies have investigated the effects of source-sink balance on resulting seed protein and oil concentration. In whole plant studies, source-sink treatments have been imposed at or near the beginning of linear seed fill (R5 or R5.5) when vegetative growth is maximized and seed number and cotyledon cell number are set (Egli et al., 1989; Munier-Jolain and Ney, 1998; Rotundo et al., 2011). Partial pod removal adjusts the source-sink balance so that seeds receive greater C and N assimilates, resulting in greater seed size and increased protein concentration at the expense of oil concentration (McAlister and Krober, 1958; Openshaw et al., 1979; Proulx and Naeve, 2009). Similar *in planta* results showed that high protein lines were less responsive than their closely-related normal protein lines to pod removal in terms of seed size and seed protein concentration increases. The decreased seed number of high protein lines (under a consistent vegetative mass) suggests the seeds were already near saturating assimilate levels (Rotundo et al., 2009). *In vitro* studies on nutrient feeding confirm that protein assimilation by the developing embryo increases linearly with the N concentration of the culture medium; however, only a small concentration of N is needed for maximal dry matter accumulation, assuming adequate C supplies (Hayati et al., 1996; Pipolo et al., 2004; Truong et al., 2013). These studies suggest that C directly influences individual seed

growth while the C-to-N ratio of supplied assimilate determines seed protein concentration. There is evidence that developing seeds in normal cultivars do not enjoy saturating C or N supply.

Source-sink treatments that reduce assimilate supply per seed, as evidenced by decreased seed size, are partial shade and partial defoliation (McAlister and Krober, 1958; Wahua and Miller, 1978; Proulx and Naeve, 2009). Partial shade affects seed composition by decreasing oil concentration and increasing protein concentration, while partial defoliation decreases protein concentration (McAlister and Krober, 1958; Proulx and Naeve, 2009). Pod removal, shade, and defoliation treatments have been able to create a range of protein concentration (343 to 388 g kg^{-1} seed) within a single variety (Proulx and Naeve, 2009). Because source-sink treatments produce a range of seed protein concentrations, we would expect that a range of amino acid balance may also be present. However, amino acid values resulting from source-sink treatments have not been reported to date.

Chapter 2 – Relationship between Soybean Seed Protein Concentration and Amino Acid Balance

Synopsis

The quality of a protein for animal growth is partially determined by the relative abundance of essential amino acids. Those essential amino acids supplied in the lowest quantity relative to the animal's requirement limit growth. Examination of soybean protein across genetic sources and environments has indicated that the abundance of potentially limiting amino acids within soybean protein may be influenced by the seed protein concentration. Our objective was to evaluate the effects of seed protein concentration on relative amino acid abundance under controlled environments in order to better understand the biological basis of this apparent relationship. This was accomplished through the use of source-sink treatments that altered seed protein concentration within environments. Increasing the source-to-sink ratio through partial pod removal and open environment treatments significantly increased seed protein; however, the resulting protein was disproportionately enriched in the amino acids glutamic acid and arginine at the expense of the limiting amino acids lysine, cysteine, methionine, threonine, and tryptophan. Defoliation treatments gave the opposite response to pod removal, resulting in a more favorable amino acid balance but with a lower seed protein concentration. Alternatively, the shade treatment increased protein concentration, but the relative concentration of the limiting amino acids was not reduced. This indicates that limiting amino acid abundance is not solely dependent on seed protein percentage and that limiting amino acids may be supplied by the vegetative tissue under C-limited conditions. The ultimate goal of soybean seed improvement is to increase yield while also increasing or maintaining seed protein concentration and the balance of the

limiting amino acids. Meeting two of these goals was achieved through the current source-sink treatments as the open environment treatment increased seed yield and protein concentration while shade increased protein concentration and maintained limiting amino acid balance. Meeting all three goals concurrently for soybean improvement was not achieved in the current experiment and may be difficult.

Introduction

Soybean is a leading source of protein meal and vegetable oil at 70% and 30% of annual global demand, respectively (USDA-NASS, 2016). Improving the concentration of both protein and oil in soybean seed is beneficial for all market stakeholders. Selecting for higher protein concentration may be hindered by a negative relationship between protein concentration and yield (Krober and Cartter, 1962; Wilcox and Zhang, 1997). Regional differences in protein concentration of soybean at the farm level are persistent (Hurburgh, 1994; Thakur and Hurburgh, 2007; Rotundo et al., 2016) and can result in processed soybean meal failing to meet the industry standard of 480 g kg^{-1} in the north-west Corn Belt states of Wisconsin, Minnesota, Nebraska, South Dakota and North Dakota (Hurburgh, 1994). Geographic separation of transport routes makes it possible for buyers to discriminate against north-west Corn Belt soybeans, producing an economic loss estimated at $0.40 a bushel or approximately $20 an acre at 50 bushels an acre yields (William W. Wilson, 2016, personal communication). This is an important economic problem considering the affected states produced 30% of U.S. soybean production in 2015 (USDA - NASS, 2015).

While both whole soybean seed and processed soybean meal commodities are evaluated by protein concentration, the ultimate nutritional value of soybean protein is determined in part by the amino acid balance (Osborne and Mendel, 1914). Amino acids are necessary for growth and productivity of animals with monogastric digestive systems and the amount of each amino acid required varies by factors such as species, sex, and growth stage (Berry et al., 1962; Fernandez et al., 1994; Boisen et al., 2000). Five amino acids which are present in the lowest concentrations in soybean protein relative to average dietary need for balanced rations are lysine, cysteine, methionine, threonine and tryptophan (Thakur and Hurburgh, 2007). Soybean quality surveys have shown that the relative abundance of these five limiting amino acids within the protein can vary between geographic areas and their enrichment is negatively correlated with crude protein (Thakur and Hurburgh, 2007; Medic et al., 2014; Miller-Garvin and Naeve, 2015). Measuring soybean protein enrichment of these five limiting amino acids would better approximate nutritional value and provide a more complete measure of value than protein concentration alone.

Factors such as genetics (Serretti et al., 1994; Zarkadas et al., 1999, 2007), nodulation and N supplementation (Krishnan et al., 2000), S supplementation (Gayler and Sykes, 1985), S and N supplementation (Paek et al., 1997, 2000; Krishnan et al., 2005), and temperature, radiation, and precipitation (Carrera et al., 2011) influence soybean seed protein amino acid balance by favoring the accumulation of some amino acids in the seed relative to others. The sulfur-containing amino acids methionine and cysteine are sensitive to the balance of S and N supplied to the seed (Gayler and Sykes, 1985; Paek et

al., 1997, 2000; Krishnan et al., 2005). Without supplemental fertility, increased protein concentration is associated with dilution of the limiting amino acids methionine, cysteine, lysine, and threonine (Serretti et al., 1994; Krishnan et al., 2005; Zarkadas et al., 2007), while glutamic acid and arginine increase (Serretti et al., 1994). These trends are also displayed across a multi-year collection of field-grown soybean samples (n= 1,805) where all five limiting amino acids as a percent of soybean protein decreased across protein levels and glutamic acid and arginine increased with increasing protein levels (Medic et al., 2014). In response to these published results, further research into the physiological basis of the relationship between protein concentration and enrichment of the five limiting amino acids is warranted. To create a wide range of protein concentrations in a set genetic background and narrow range of environments, we imposed source-sink treatments that have been shown in previous work to lower (partial defoliation) and raise (partial pod removal, partial shade) the protein concentration of soybean seed (McAlister and Krober, 1958; Wahua and Miller, 1978; Openshaw et al., 1979; Proulx and Naeve, 2009). An open environment treatment that would allow greater overall resource availability to whole plants was also added as light augmentation with artificial lighting has been shown to increase N_2 fixation during seed fill (Lawn and Brun, 1974), and may therefore increase seed protein concentration (Sinclair and de Wit, 1975; Egli and Bruening, 2007; Zhao et al., 2014). Using source-sink treatments to change seed protein concentrations and investigate effects on amino acid quality may help clarify the basis for the changes and possible trade-offs involved. Our objectives were to i) determine whether the relative abundance of amino acids within soybean seed

treatments can vary within limited environments and soybean genotypes, and ii) examine possible relationships between protein concentration and amino acid balance and any differences in the relationship between treatments.

Materials and Methods

Environments

Soybean field studies were conducted in three growing seasons at University of Minnesota locations in St. Paul [44° 99' N, 93° 17' W; Waukegan silt loam (2-6% slopes, fine-silty over sandy or sandy-skeletal, mixed, superactive, mesic Typic Hapludolls)] and Waseca [44° 07' N, 93° 52' W'; Webster clay loam (0-2 percent slopes, fine-loamy, mixed, superactive, mesic Typic Endoaquolls)]. Field preparation followed University of Minnesota recommendations regarding tillage and fertilization in soybean following corn. Soybean was planted on 15 May 2013, 15 May 2014, and 23 May 2015 in St. Paul and 16 May 2013, 20 May 2014, and 12 May 2015 in Waseca. The 2014 Waseca location was removed from the experiment due to hail damage. The remaining five location x year combinations represented in this study will be referred to as unique environments: STP13, WAS13, STP14, STP15 and WAS15. Two commercial varieties were seeded in each environment at a rate of 345,000 seeds ha^{-1}. In STP13, WAS13, STP14 the varieties were Pioneer brand P92Y22 and P92Y32 (RM 2.2 and 2.3). In STP15 and WAS15, Pioneer brand varieties were P22T41R2 and P22T61R (RM 2.4 and 2.6). It was necessary to change varieties due to seed availability. All varieties were modern elite

cultivars selected to have similar maturities and protein concentrations so only a small influence of cultivar on amino acid balance is expected (Zarkadas et al., 2007). Soybean was seeded into 3-meter long plots with four 76-cm rows. The trials were a randomized complete block design with four replications. Standard agronomic practices for weed control were used and escapes were manually removed.

Treatments

Treatments for the study are referred to as "source-sink" because they change the ratio of vegetative nutrient supply (source) to reproductive demand (sink) through targeted manipulation. General treatment categories were different levels of pod removal, defoliation, shade, and resource augmentation adapted from previous work (Proulx and Naeve, 2009). All treatments were imposed at the beginning of the seed fill reproductive stage (R5) except for a second set of defoliation treatments that were conducted at the full seed reproductive stage (R6). Pod removal was conducted at two levels to reduce yield by approximately 40 and 70% relative to the control to approximate yield reductions due to defoliation and shade, respectively. This was accomplished by hand-removal of pods from the bottom three plant nodes and associated branches, then continuing to remove pods in an every-other node or remove three and leave one node pattern in the 40% and 70% pod removal treatments, respectively (Figure 1). Pods were removed from plants in one-meter length of plot rows two and three. Any additional pods that formed after initial pod removal were removed during treatment rechecks. Defoliation was accomplished in a similar manner by carefully removing two leaflets of

every trifoliate on plants in the one-meter treatment area of rows two and three, leaving one intact trifoliate on each petiole. The remaining length of rows two and three and all of the border rows received a less precise hand removal of leaves to mimic the two-thirds defoliation treatment in the harvest area. Shade was imposed by hanging a black shade cloth (Gempler's, Madison, WI) rated for 80% irradiation reduction. The shade cloth was suspended 0.5 m above the harvest rows by wire tensioned between fence posts. The open environment treatment involved removing all plants in the treated plot, except for the one-meter harvest length of rows two and three, by clipping them off at the base; this resulted in a plot that was mostly open space.

When soybean plants had reached harvest maturity (R8) the one-meter lengths of the treatment rows were hand-cut and machine-threshed. Samples were then taken to the lab for yield determination and moisture measurement, and were cleaned to remove split seeds and minor debris. Seed counts were performed on a DuBois Model 2500 High Speed Seed Counter (DuBois Engineering Company, Kansas City, Missouri) and samples were re-weighed to determine seed size. Seed quality attributes were determined by the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories. Specific constituents measured were crude protein by combustion analysis method, crude oil by ether extraction, and complete amino acid profile using AOAC Official Methods 982.30, 990.03 and 934.01, respectively (AOAC International, 2016). Yield, protein, and oil values are provided on a 130 g kg^{-1} moisture basis. Amino acid data are shown after conversion from hydrated amino acid weight, as reported, to the dehydrated weight actually found in the peptide form, which is less one water molecule

per amino acid residue (Mosse, 1990). Because all amino acids have unique molecular weights this ratio is also unique (with the exception of the structural isomers isoleucine and leucine). To investigate the main research question of amino acid balance, all amino acid values were divided by the total 18 amino acids.

Analysis

The following mixed-effects linear model was fit using JMP Pro v 11.2.1 (2013 SAS Institute Inc.)

$$y_{ijkl} = trt_i + env_j + (trt \times env)_{ij} + variety(env)_{k(j)} + block(env)_{l(j)} + (trt \times variety)(env)_{ik(j)} + e_{ijkl}$$

where trt_i is the fixed effect of the *i*th treatment, env_j is the random effect of the *jth* environment, $variety_k$ and $block_l$ are the random effects of the *k*th variety and *l*th block nested within the *j*th environment, respectively, (*trt x variety*) is the random interaction between the *i*th treatment and *k*th variety nested within the *j*th environment, and e_{ijkl} is the random error term associated with observation y_{ijkl}. If trt_i was significant according to its *F*-statistic ($P < 0.05$), the significance of the random variables was then tested using the likelihood ratio test (LRT) making use of the restricted effects maximum likelihood (REML) value. Specifically, if the value of $-2*(loglik_{null} - loglik_{alt})$ is greater than the critical value given by a chi-square distribution with a single degree of freedom at $\alpha = 0.05$, where $loglik_{null}$ is the residual log-likelihood of the model without the random effect being tested and $loglik_{alt}$ is the residual log-likelihood of the model with the random effect being tested, then the random effect is considered significant and enters the model

(Littell et al, 2006). The LRT is performed sequentially for all random effects, with the most significant random effect entering the model at each iteration.

The fitted variance components were then estimated along with the LSMEANS value for treatments (2013 SAS Institute Inc.). Dunnett's test was used to compare all treatments to the control (Littell et al., 2006). For variables with significant environment x treatment interaction or treatment x variety interaction, a LS MEANS graphical plot was utilized to investigate the nature of interactions. Lastly, sample attributes were indexed to their within-block control to test relationships between protein concentration and the other seed yield and quality attributes, especially amino acid balance. Plotting was performed in R version 4.8.3 (R Development Core Team, 2014) and the strength of correlations are shown by Pearson's correlation coefficient.

Results

Environment

Growing season conditions for the five environments are displayed in Table 1. Seasonal average temperature was about 19°C in all environments which approximated the 30-year average for Waseca but was about 0.5°C cooler than normal for St. Paul. Season precipitation totals were slightly above average for all environments except WAS15 which received a major increase (approx. 45%) in rainfall. Despite the precipitation difference between the two Waseca environments, the control yields of 4.31 and 4.80 Mg ha^{-1} were similar in WAS13 and WAS15, respectively. In St. Paul the control yields showed more variability between environments with yields of 4.16, 5.83,

and 3.64 Mg ha^{-1} in STP13, STP14, and STP15, respectively. The local weather conditions, especially the distribution of rainfall patterns within each month, are critical to explain these differences. Although STP14 and STP15 were the highest and lowest yielding environments, respectively, they are the only environments where the open environment treatment did not significantly increase yield compared to the control. This outcome suggests that late-season intercrop competition did not significantly limit yields in these environments. Because treatments were initiated at beginning seed fill this suggests that plants under the open environment treatment did not benefit from increased resources in the period from beginning seed fill to maturity in STP14 and STP15.

Significance of model terms

An analysis of yield characteristics (Table 2) showed significant effects of treatment as well as environment, block, and genotype. Significant treatment by environment interactions were observed for yield, seed number, threonine, leucine, and cysteine. Graphical investigation showed that these interactions were primarily due to similar values between the open environment treatment and the control treatment in STP14 and STP15 compared to large differences in the other three environments (see appendix for example). The interaction of environment and variety with treatment resulted in magnitude differences, not changes in the direction between the treatment mean and control. Therefore, treatment means are displayed as average values across environments and varieties (Table 3). Dunnett's minimum significant difference is provided to compare treatment values to the control ($P = 0.05$).

Major yield characteristics

Values for yield, seed number, seed size, protein, oil, and the numerical sum of protein and oil (P+O) are shown by treatment averaged across the five environments and two genotypes within each environment (Table 3). Yield, seed number, and seed size are related measurements. The combination of seed number and seed size, measured on an area basis, determines yield. On average, the open environment treatment significantly increased yield and seed size. Yield was decreased by partial defoliation at R5, as well as by pod removal and shade. Seed number was significantly reduced and seed size increased by pod removal, seed size was significantly reduced by defoliation, and both seed number and seed size were reduced under shade. The open environment treatment increased the protein concentration, maintained oil concentration, and increased P+O concentration. Pod removal and shade treatments also increased protein concentration, but in contrast with the open environment treatment, decreased oil concentration. Shade decreased oil concentration at twice the magnitude of severe pod removal. This large quantitative decrease shade imposed on oil meant that shade did not increase P+O relative to the control, while pod removal did increase P+O. Defoliation was the only treatment found to decrease protein compared to the control. Defoliation produced no change in oil concentration and therefore P+O was reduced by defoliation. Across all treatments, protein concentration was both increased and decreased relative to the control; however, no treatment increased oil concentration above control levels.

Additionally, P+O changes were driven by the larger constituent, protein, except under the shade treatment.

Amino acid abundance

Treatment means for individual amino acids as percent of the total 18 amino acids (TAA) are shown in Table 3. The sub-grouping of the amino acids is by metabolic precursor (Buchanan et al., 2000). The control totals range from the greatest TAA proportion of 31.2% for the α-ketoglutarate group (arginine, glutamic acid, proline) to 2.67% for 2-ribose 5-phosphate (histidine). The most important metabolic group from a nutritional standpoint is the oxaloacetate group as it contains three of the five limiting amino acids in soybean: lysine, methionine, and threonine. Oxaloacetate-derived amino acids are the second most abundant grouping at 28.9% of the total amino acids in the control treatment. Pod removal treatments had enrichment in α-ketoglutarate amino acids by 0.73 and 0.96 percentage points in moderate pod removal and severe pod removal, respectively. Arginine and glutamic acid are the only individual amino acids that increased in abundance under pod removal; the other 16 amino acids were less abundant or unchanged compared to the control. The largest decreases in the severe pod removal treatment were found in the oxaloacetate and pyruvate groups with 0.39 and 0.24 percentage point decreases, respectively. Shade treatment showed a similar pattern of arginine increase when compared to the control but the treatment did not affect glutamic acid. Shade also differed from pod removal in that it increased abundance of the S-containing amino acids cysteine and methionine, as well as histidine when compared to

the control. Shade decreased the pyruvate amino acid group the most at 0.34 percentage points and increased the α-ketoglutarate group 0.42 percentage points. The earlier defoliation treatment (R5) showed the opposite result of pod removal with an increased or maintained abundance of all amino acids except for significant decreases in arginine and glutamic acid (0.14 and 0.27 percentage points, respectively). The open environment treatment showed some decreases in amino acids such as the limiting amino acids cysteine, methionine, lysine, and threonine. Open environment increased arginine and glutamic acid numerically, although this was not significant for the individual amino acids or the overall α-ketoglutarate group.

Relationships between protein concentration and amino acid balance

The primary focus of this work was to evaluate the effect of seed protein concentration on amino acid balance. To observe this relationship, quality attribute data were indexed to the control within block to remove effects of variety, block, and environment. Crude protein was plotted as the explanatory variable against the other quality attributes to characterize the ability of crude protein to predict each attribute. In the means table (Table 3) shade treatment seemed to have a unique relationship with crude protein and amino acid balance as an increase in protein concentration was observed but limiting amino acid balance was unchanged. Therefore, the correlation analysis was done twice, both with and without shade data points, to quantify the unique yield responses due to the shade treatment. The strength and direction of the relationship, shown as Pearson's correlation coefficient, is provided in Table 4. Crude protein was

significantly correlated with the main yield characteristics calculated both with and without the shade treatment data points. Protein was negatively correlated with yield, seed number, and oil and positively correlated with seed size and P+O (Table 4). Crude protein showed the same relationship with most yield characteristics when the shade treatment data points were added, except the strong positive relationship between seed protein and seed size was confounded by the small seed size resulting from the shade treatment (appendix). Additionally, across all treatments except shade, crude protein was a significant predictor of enrichment in 15 of 18 amino acids (Table 4). The relationship between protein concentration and enrichment of most amino acids was negative. The three amino acids that increased in abundance with increased seed protein are the non-essential amino acids arginine, glutamic acid, and aspartic acid. Therefore, a strong negative relationship between protein and abundance of the five limiting amino acids ($r = -0.79$) and the ten essential amino acids ($r = -0.83$) was observed. When the shade treatment data points were included, the negative correlation became less pronounced between protein concentration and enrichment in the five limiting amino acids, the essential amino acid histidine, and all ten essential amino acids (Figure 2 - Figure 9). In terms of non-essential amino acids, adding the shade data points didn't change the correlation between protein and enrichment of arginine (Figure 10) but the correlation with glutamic acid enrichment was decreased (Figure 11).

Discussion

Major yield characteristics

Altering the source-sink relationship created a range of seed protein concentration (324–376 g kg^{-1}) that was largely consistent with the range of 343-388 g kg^{-1} reported from previous use of concurrent source-sink treatments (Proulx and Naeve, 2009). The decrease in protein concentration from defoliation and the increase in protein concentration from pod removal and shade agreed with published results (McAlister and Krober, 1958; Wahua and Miller, 1978; Openshaw et al., 1979; Proulx and Naeve, 2009). Oil concentration was more constant across treatments with small decreases observed under pod removal treatments and a large decrease from shade treatment. The sum of protein and oil concentrations was determined by protein concentration, except under shade conditions where the low oil concentration more than offset the positive change in protein concentration. Oil concentration was not increased in this study which may suggest C conditions that are close to saturation. *In vivo* studies on C:N ratios have revealed that oil is less variable across C:N ratios than protein because decreased C:N ratios favor higher protein at the expense of the carbohydrate concentration of the seed, not the oil concentration (Pipolo et al., 2004; Truong et al., 2013). Under treatments with normal light conditions (all treatments except shade) N is the likely driver of seed quality in terms of P+O concentration. Shade seems to impose C-limited conditions based on resulting decreased oil concentrations; the total yield of oil decreased from 0.81 to 0.30 Mg ha^{-1} (63% decrease) while protein decreased from 1.54 to 0.76 Mg ha^{-1} (50% decrease). Therefore, it can be argued that the high protein concentration under shade

treatment results from reduced oil deposition due to a greater decrease in supplied C than supplied N. The concept of C and N limitations to oil and protein yield formation is based on current reported findings in embryo culture (Truong et al., 2013) and helps contextualize the results of these source-sink treatments.

Additionally, research on high protein lines often shows that these lines yield less than normal protein lines (Serretti et al., 1994; Rotundo et al., 2009b). This is due to reduced seed set by high protein lines, which affords greater N reserves per seed. Pod removal on high protein lines gave less response in terms of protein concentration increases, suggesting the seeds were already supplied high N levels (Rotundo et al., 2009b). These results are consistent with the increased protein concentration resulting from pod removal in this study. They also provide evidence for N supply per seed being an important driver in the C:N ratio which results in protein concentration changes. Across all treatments, weak negative correlations between protein and yield were observed, as has been commonly reported (Krober and Cartter, 1962; Wilcox and Zhang, 1997; Rotundo et al., 2009). However, the open environment treatment increased seed yield while also increasing seed percent protein (Table 3). An examination of individual treatments revealed that open environment produced a positive relationship between protein and seed yield ($r = 0.58$). Our result of increased seed yield under open environment treatment is consistent with the findings of Lawn and Brun under light enhancement, although they did not observe a concomitant increase in seed protein concentration (Lawn and Brun, 1974), as was noted here.

Amino Acids

The relative concentrations of individual amino acids for control samples were similar to those reported previously (Zarkadas et al., 1999, 2007) with slightly lower levels of methionine, cysteine, and serine. The treatment mean values reported here support the hypothesis that source-sink treatments in soybean known to affect seed protein concentration also change the amino acid balance, an important nutritional component of the protein. Pod removal significantly ($P < 0.05$) increased protein concentration and the protein was more abundant in arginine and glutamic acid (Table 3). This is in agreement with previous work which showed high protein soybean lines have increased concentration of arginine and glutamic acid compared to control lines (Serretti et al., 1994).

Glutamate is one of the main molecular transporters of N to the developing embryo (Layzell and Larue, 1982; Rainbird et al., 1984), and arginine has the highest nitrogen-to-weight ratio of all amino acids. The combined evidence suggests that increasing the source-sink ratio results in the maternal plant supplying the developing embryo with an increased N supply and the supply may be more concentrated in the molecular forms of arginine and glutamic acid. Additionally, metabolic activities of the developing seed itself may increase the balance of arginine and glutamic acid under increased source-sink ratio. Glutamic acid is involved in the capture of free N within the seed and is a major component of soybean seed storage proteins (Woo Min et al., 2015). The seed storage protein shown to be concentrated in high protein lines is the β-conglycinin protein which is high in glutamic acid, but low in the sulfur-containing limiting amino acids (Yaklich,

2001). Increased concentration of β-conglycinin protein would be expected from treatments that increase N reserves and seed protein concentration (pod removal, open environment) as increased N fertilization has delivered this result (Paek et al., 1997). Arginine leads all amino acids with 18% of total cotyledon N and 50% of the N found in the free amino acid pool (Micallef and Shelp, 1989). Transgenic seeds show increased arginine as a free amino acid (Takahashi et al., 2003; Kita et al., 2010) so this mechanism of arginine deposition cannot be ruled out as a factor in its increased amino acid balance. These speculations on the relationship between whole plant physiology and seed amino acid balance should be interpreted with caution. No direct measurement of *in situ* seed metabolism was made during seed development under the source-sink treatments. While the pod removal and the open environment treatments increased arginine and glutamic acid, all the limiting amino acids except tryptophan were significantly reduced (Table 3). This is consistent with previous reports that higher percent seed protein lines are generally lower in the S-containing amino acids cysteine and methionine (Zarkadas et al., 2007), and some high protein lines show a dilution of threonine, methionine, and lysine (Serretti et al., 1994).

In the correlation analysis without shade treatments, strong positive relationships between protein and glutamic acid ($r = 0.76$), arginine ($r = 0.89$), and aspartic acid ($r = 0.28$) were observed, while an equally strong negative relationship was apparent between protein and the five limiting amino acids ($r = -0.79$). The direction of the relationships agreed with reported trends from surveys of farmer-grown soybean (Thakur and Hurburgh, 2007; Medic et al., 2014; Miller-Garvin and Naeve, 2015). However, the

current study gave stronger correlations due to the relatively narrow range of environments and varieties tested. When shade is included, the weaker correlations in Figure 2 through Figure 11 demonstrate that shade didn't induce the same response in the five limiting amino acids expected from its protein concentration. The shade treatment, producing C-limited conditions, may mimic field environments where seeds develop under cloudy conditions leading to high seed protein concentration with maintained levels of limiting amino acids. This lack of supplied C separates whole plant source-sink conditions imposed by the shade treatment from the other treatments that resulted in higher protein concentration. Additionally, because the shade treatment may have decreased N_2 fixation (Lawn and Brun, 1974) the seed likely received a greater proportion of N remobilized from the vegetative tissues.

Therefore, those amino acids that are maintained in the shade treatment could be supplemented by direct transfer from vegetative tissues to seed during senescence. In other words, the amino acids maintained under shade may not be as dependent on C supply during seed filling as other amino acids. All of the limiting amino acids were maintained under shade, with increases for the S-containing amino acids compared to the control. This supports a possible role for vegetative reserves to support protein quality through enhancing the relative amounts of the limiting amino acids, which may not be as C-dependent. On the other hand, conditions that limit C fixation appear to limit some amino acids. The shade treatment resulted in the lowest amino acid balance of amino acids derived from pyruvate, a product of the first step of glycolysis.

Conclusions

Source-sink treatments significantly altered the composition of soybean seed, especially protein concentration. The amino acid balance of the protein fraction was also affected. When seed protein concentration was increased by treatments under normal light conditions (pod removal and open environment), the non-essential amino acids arginine, glutamic acid, and aspartic acid were favored. When seed protein was increased through imposition of shade during seed-fill, no reduction of the limiting amino acids was observed. This suggests that the limiting amino acids of soybean protein may still be enriched at high protein concentrations when photosynthate is limiting to seed development. Decreasing seed protein concentration through the use of defoliation treatments preserved or increased the concentration of limiting and essential amino acids. Overall, protein quality does not seem to be completely linked with protein concentration, as has been suggested by surveys of farmer-grown soybeans, but the concentration of amino acids in the protein was only improved by treatments that decreased the source-sink ratio (shade and defoliation). Based on these results, increasing soybean yields while also improving seed protein concentration and amino acid balance may prove difficult.

Tables and Figures

Table 1. Average temperature and precipitation by month for five environments in Minnesota. Data are from weather stations onsite at experimental research centers.

	WAS13		WAS15		STP13		STP14		STP15	
	Value	Dep.†	Value	Dep.	Value	Dep.	Value	Dep.	Value	Dep.
Temp‡	----------°C----------									
May	13.0	(1.7)	14.4	(0.2)	12.6	(2.6)	13.8	(1.4)	13.8	(1.3)
June	19.6	(0.7)	20.2	(0.2)	18.9	(1.6)	21.1	0.5	19.8	(0.7)
July	22.1	0.0	21.4	(0.7)	21.8	(1.1)	21.8	(1.1)	21.9	(1.1)
August	20.9	0.2	19.8	(0.9)	21.7	0.2	22.2	0.7	20.1	(1.4)
Sept.	17.9	1.5	19.9	3.5	18.2	1.1	16.1	(1.0)	18.8	1.8
Mean	18.7	(0.1)	19.2	0.3	18.7	(0.8)	19.0	(0.5)	18.9	(0.6)
Precip§	----------mm----------									
May	164	61	121	18	183	82	90	(11)	125	25
June	169	35	194	59	186	67	234	115	84	(35)
July	134	18	188	71	81	(41)	69	(52)	157	35
August	53	(60)	152	40	38	(64)	98	(3)	71	(30)
Sept.	49	(39)	149	61	33	(44)	56	(21)	97	20
Total	569	14	804	249	520	1	547	28	535	15
SFP Temp.¶	----------°C----------									
Daily Max	27.9		25.3		28.3		23.8		24.2	
Daily Min	15.8		13.6		16.8		14.7		14.8	
Daily Avr	21.9		19.5		22.5		19.3		19.5	
SFP Precip††	----------mm----------									
Total	30		207		19		113		97	

† Positive (negative) departure from location 30-year average 1986-2015.

‡ Average temperature by month.

§ Total precipitation by month, STP14 environment received 20 mm irrigation on 8/28/2014 which is included in the totals

¶ Daily maximum, daily minimum, and daily average temperatures during the approximate seed-filling period (15 Aug.-14 Sept.).

†† Total precipitation during the approximate seed-filling period

Table 2. Part 1 of 2. Significance of the fixed effect, treatment, and random effects given by the P values from the Likelihood Ratio Test for all yield characteristics. Quality data are from seed produced under various source-sink treatments designed to change seed protein concentration and represent five MN environments.

	Yield Characteristics					
	Yield	Seed Number	Seed Size	Protein	Oil	P+O
Source of Variation						
Treatment (Trt)	***	***	***	***	***	***
Environment (Env)	***	***	†ns	***	***	***
Trt x Env	***	***	ns	ns	ns	ns
Block	***	***	***	***	***	*
Variety	*	***	***	*	***	**
Variety x Trt	ns	***	***	***	ns	***

	AA group 1 - Oxaloacetate						AA group 2-Ribose 5-phosphate
	Aspartic Acid	Isoleucine ¶	Lysine §	Methionine §	Threonine §	Total 1	Histidine ¶
Source of Variation							
Treatment (Trt)	***	***	***	***	***	***	***
Environment (Env)	***	***	***	***	***	***	***
Trt x Env	ns	ns	ns	ns	*	ns	ns
Block	**	***	ns	***	***	***	*
Variety	ns	***	ns	***	ns	ns	ns
Variety x Trt	*	ns	***	ns	ns	**	***

	AA group 3 - Pyruvate				AA group 4 - Phsphoenolpyruvate + Erythrose 4-phosphate			
	Alanine	Leucine ¶	Valine ¶	Total 3	Phenylalanine ¶	Tryptophan §	Tyrosine	Total 4
Treatment (Trt)								
Environment (Env)	***	***	***	***	ns	***	***	***
Trt x Env	***	***	***	***	***	***	***	***
Block	ns	***	ns	ns	ns	ns	ns	ns
Variety	ns	**	***	***	***	ns	***	***
Variety x Trt	*	***	ns	*	***	ns	ns	ns
Treatment (Trt)	ns	ns	ns	***	ns	ns	ns	ns

Table 2. Part 2 of 2.

	AA group 5 - 3-Phosphoglycerate				AA group 6 - α-Ketoglutarate			
	Cysteine §	Glycine	Serine	Total 5	Arginine	Glutamic Acid	Proline	Total 6
Source of Variation								
Treatment (Trt)	***	***	ns	***	***	***	**	***
Environment (Env)	***	***	***	***	***	***	***	***
Trt x Env	*	ns	ns	ns	ns	ns	ns	ns
Block	***	***	***	***	***	*	ns	ns
Variety	**	***	**	ns	ns	ns	**	ns
Variety x Trt	***	ns	ns	ns	***	*	ns	***

	Sum 5 Limiting AA §	Sum 10 Essential AA ¶
Source of Variation		
Treatment (Trt)	***	***
Environment (Env)	***	***
Trt x Env	**	ns
Block	**	***
Variety	**	ns
Variety x Trt	ns	**

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.
† ns, nonsignificant.
‡ Block, Variety, and Variety x Treatment nested within Environment
§ The five limiting amino acids in soybean protein are lysine, methionine, cysteine, threonine, tryptophan
¶ The ten essential amino acids are lysine, methionine, cysteine, threonine, tryptophan, isoleucine, leucine, valine, histidine, phenylalanine

Table 3. Part 1 of 3. Seed characteristics for a collection of soybean seed produced using source-sink treatments and representing the average values of five MN environments and two varieties per environment. Dunnett's value represents the minimum significant difference from the control (*P* = 0.05).

Treatment	Yield	Seed Number	Seed Size	Protein	Oil	P+O	Sum 18 Amino Acids
	Mg ha^{-1}	seeds m^{-2}	g 100 seed^{-1}	g kg^{-1}			
Control	4.55	2750	16.8	339	179	518	327
Open environment	5.43*	3030 ns	18**	345*	182 ns	527***	330 ns
Defoliation R5	3.35**	2310 ns	14.7***	324***	181 ns	506***	312***
Defoliation R6	3.86 ns†	2600 ns	15***	326***	183 ns	509**	314***
Pod Removal 40%	2.51***	1270***	19.9***	358***	172***	530***	344***
Pod Removal 70%	1.8***	920***	19.7***	365***	163***	528***	348***
Shade 80%	2.03***	1800***	11.6***	376***	149***	524 ns	357***
Multiple comparison	Mg ha^{-1}	seeds m^{-2}	g 100 seed^{-1}	g kg^{-1}			
Dunnett's MSD (0.05)‡	0.82	480	0.9	6	4	6	7

	AA group 1 – Oxaloacetate ‡						AA group 2-Ribose 5-phosphate
Treatment	Aspartic Acid	Isoleucine ¶	Lysine §	Methionine §	Threonine §	Total 1	Histidine ¶
Control	11.59	4.86	7.00	1.47	3.93	28.86	2.76
Open environment	11.58 ns	4.87 ns	6.95 *	1.44 ***	3.90 *	28.74 **	2.74 ns
Defoliation R5	11.56 ns	4.90 **	7.09 ***	1.45 ns	3.98 **	28.98 **	2.78 ns
Defoliation R6	11.56 ns	4.88 ns	7.08 ***	1.47 ns	3.98 *	28.97 **	2.77 ns
Pod Removal 40%	11.59 ns	4.81 ***	6.88 ***	1.42 ***	3.85 ***	28.55 ***	2.73 ns
Pod Removal 70%	11.60 ns	4.78 ***	6.86 ***	1.41 ***	3.82 ***	28.47 ***	2.73 *
Shade 80%	11.61 ns	4.73 ***	6.98 ns	1.50 **	3.91 ns	28.72 ***	2.86 ***
Multiple comparison							
Dunnett's MSD (0.05)	0.04	0.03	0.05	0.02	0.04	0.09	0.03

Table 3. Part 2 of 3.

Treatment	AA group 3 - Pyruvate				AA group 4 – Phosphoenol.+Ery.-4-phosphate			
	Alanine	Leucine ¶	Valine ¶	Total 3	Phenylalanine ¶	Tryptophan §	Tyrosine	Total 4
Control	4.09	7.92	5.06	17.08	5.42	1.01	3.74	10.18
Open environment	4.07 ns	7.94 ns	5.06 ns	17.08 ns	5.45 ns	1.02 ns	3.75 ns	10.23 ns
Defoliation R5	4.16 ***	7.97 ns	5.10 ***	17.23 ***	5.43 ns	1.01 ns	3.81 ***	10.25 ns
Defoliation R6	4.14 ***	7.94 ns	5.07 ns	17.15 ns	5.42 ns	1.03 ns	3.79 **	10.25 *
Pod Removal 40%	4.02 ***	7.88 ns	5.02 *	16.93 ***	5.44 ns	0.97 ns	3.70 *	10.11 ns
Pod Removal 70%	4.00 ***	7.85 **	4.99 ***	16.84 ***	5.41 ns	0.95 *	3.67 ***	10.03 ***
Shade 80%	4.04 ***	7.73 ***	4.96 ***	16.73 ***	5.39 ns	1.05 ns	3.68 ***	10.12 ***
Multiple comparison		ns						
Dunnett's MSD (0.05)	0.02	0.06	0.03	0.08	0.04	0.05	0.04	0.08

Treatment	AA group 5 - 3-Phosphoglycerate				AA group 6 - α-Ketoglutarate			
	Cysteine §	Glycine	Serine	Total 5	Arginine	Glutamic Acid	Proline	Total 6
Control	1.43	4.12	4.42	9.97	7.70	18.45	5.02	31.17
Open environment	1.37 *	4.09 ns	4.43 ns	9.9 ns	7.79 ns	18.53 ns	5.00 ns	31.33 ns
Defoliation R5	1.4 ns	4.17 ***	4.44 ns	10.01 ns	7.56 **	18.19 ***	5.00 ns	30.75 ***
Defoliation R6	1.43 ns	4.17 ***	4.43 ns	10.03 ns	7.57 *	18.23 ***	5.02 ns	30.82 ***
Pod Removal 40%	1.36 *	4.04 ***	4.38 ns	9.79 ***	8.10 ***	18.74 ***	5.06 ns	31.90 ***
Pod Removal 70%	1.35 **	4.03 ***	4.44 ns	9.81 ***	8.29 ***	18.82 ***	5.02 ns	32.12 ***
Shade 80%	1.51 ***	4.07 ***	4.41 ns	9.99 ns	8.21 ***	18.41 ns	4.97 *	31.58 ***
Multiple comparison								
Dunnett's MSD (0.05)	0.06	0.03	0.07	0.07	0.11	0.14	0.05	0.19

Table 3. Part 3 of 3.

Treatment	Sum 5 Limiting AAs §	Sum 10 Essential AAs ¶
Control	14.85	40.87
Open environment	14.68 *	40.74 ns
Defoliation R5	14.93 ns	41.11 ***
Defoliation R6	15.00 *	41.08 **
Pod Removal 40%	14.48 ***	40.37 ***
Pod Removal 70%	14.39 ***	40.14 ***
Shade 80%	14.95 ns	40.61 ***
Multiple comparison		
Dunnett's MSD (0.05)	0.15	0.16

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.
† ns, nonsignificant.
‡ Amino acids displayed as percent of 18 amino acids
§ The five limiting amino acids in soybean protein are lysine, methionine, cysteine, threonine, tryptophan
¶ The ten essential amino acids are lysine, methionine, cysteine, threonine, tryptophan, isoleucine, leucine, valine, histidine, phenylalanine

Table 4. Correlation between protein and quality attributes for all indexed data points. Correlations are shown both with and without the shade treatment observations. Direction and strength of correlations are indicated by the Pearson's correlation coefficient (r). All starred relationships are significant at *P* < 0.001.

	All Treatments Except Shade		With Shade	
	Protein corr.	Sig diff than 0	Protein corr.	Sig diff than 0
Quality characters				
Yield	-0.40	***	-0.54	***
Seed Number	-0.60	***	-0.58	***
Seed Size	0.83	***	0.07	ns
Oil	-0.58	***	-0.74	***
Protein + Oil	0.77	***	0.69	***
Five Limiting AAs				
Lysine	-0.85	***	-0.67	***
Cysteine	-0.41	***	0.06	ns
Methionine	-0.41	***	-0.07	ns
Threonine	-0.78	***	-0.63	***
Tryptophan	-0.23	***	-0.07	ns
Other Essential AAs				
Histidine	-0.61	***	0.18	***
Isoleucine	-0.50	***	-0.62	***
Leucine	-0.53	***	-0.70	***
Phenylalanine	0.01	ns	-0.08	ns
Valine	-0.46	***	-0.58	***
Non-essential AAs				
Alanine	-0.79	***	-0.73	***
Arginine	0.89	***	0.90	***
Aspartic Acid	0.28	***	0.29	***
Glutamic Acid	0.76	***	0.52	***
Glycine	-0.68	***	-0.61	***
Proline	0.02	ns	-0.07	ns
Serine	-0.04	ns	-0.07	ns
Tyrosine	-0.56	***	-0.55	***
Amino Acids Sums				
5 Limiting AAs	-0.79	***	-0.44	***
10 Essential AAs	-0.83	***	-0.71	***

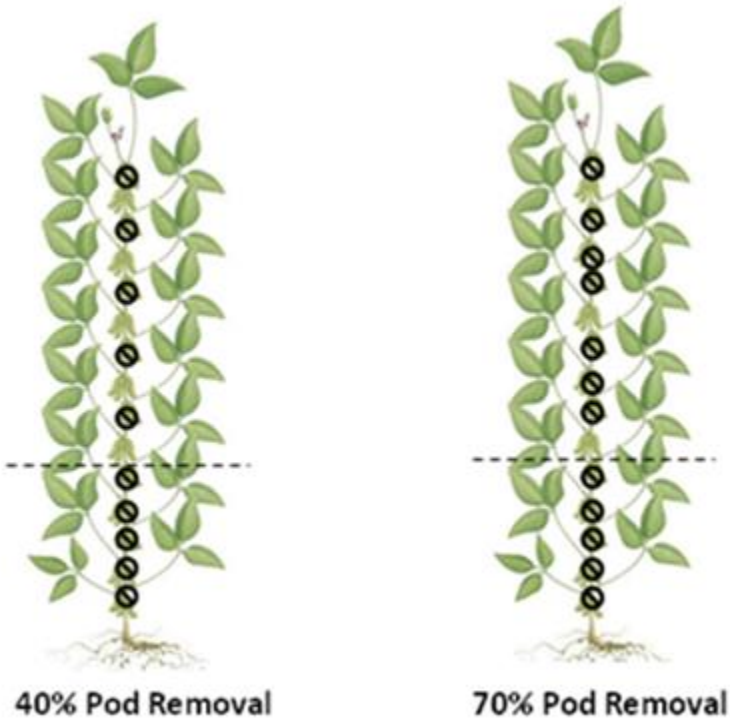

Figure 1. Pod removal patterns to achieve 40% and 70% yield reduction relative to control. Pods were removed from the indicated internodes and their associated branches.

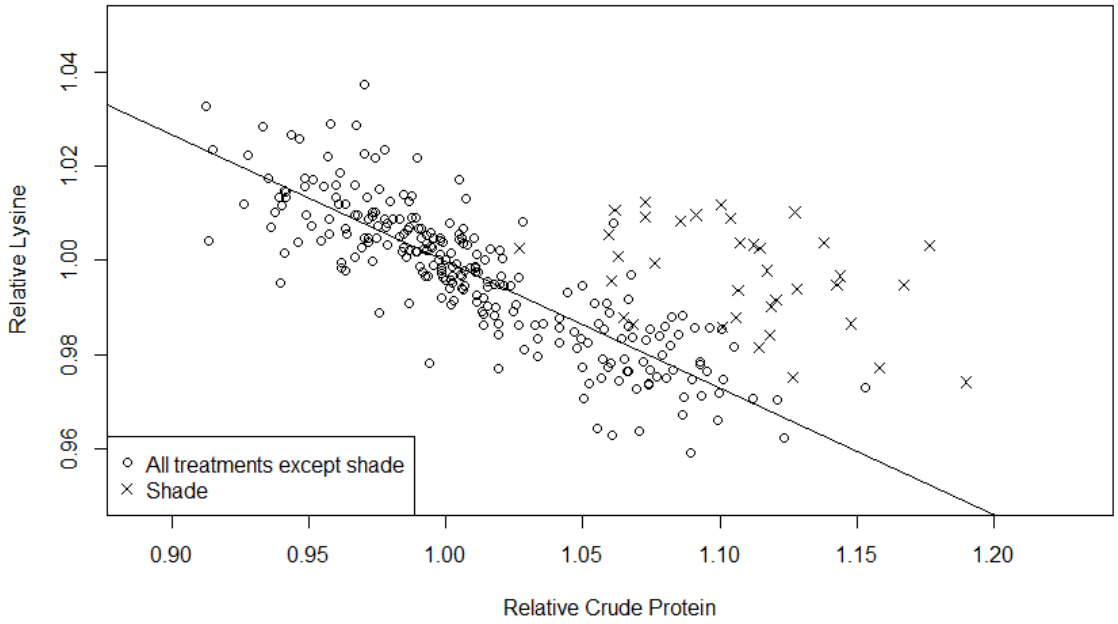

Figure 2. Relationship of relative lysine with relative crude protein across 5 environments and 4 varieties. Line is best fit for all data points except shade.

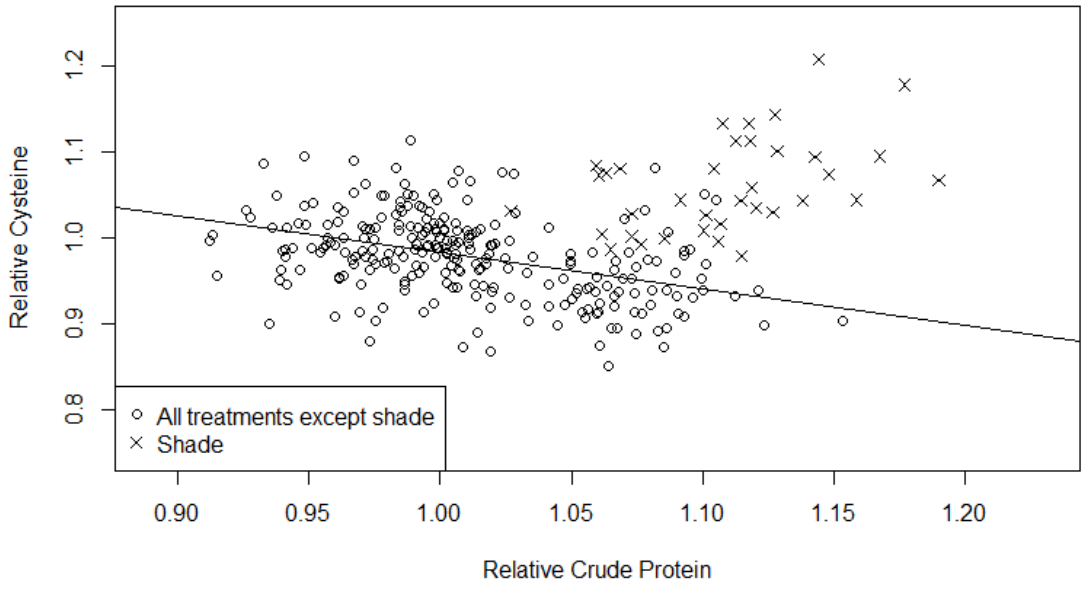

Figure 3. Relationship of relative cysteine with relative crude protein across 5 environments and 4 varieties. Line is best fit for all data points except shade.

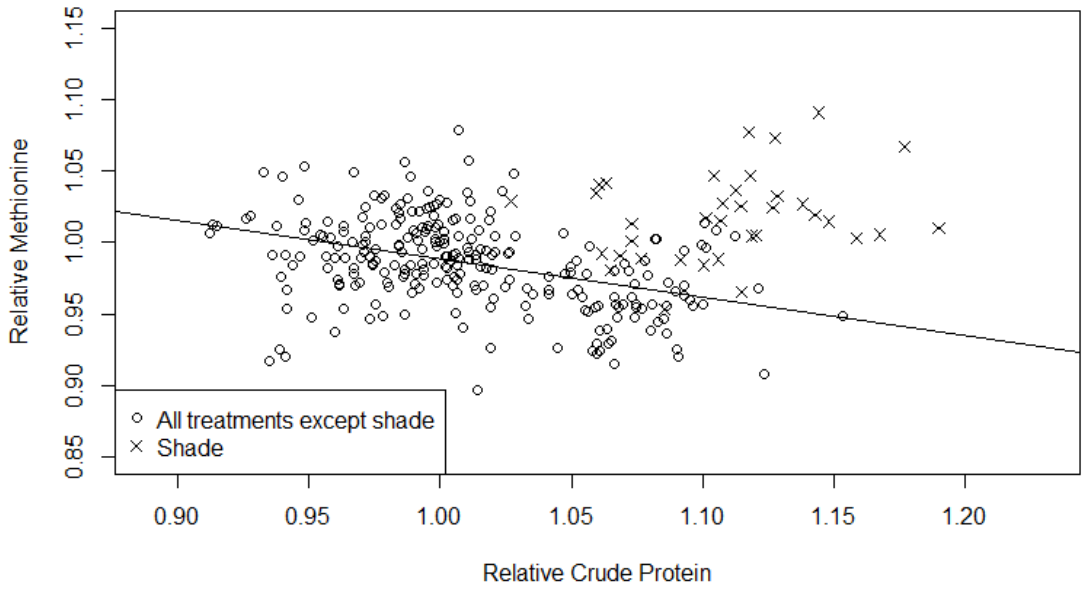

Figure 4. Relationship of relative methionine with relative crude protein across 5 environments and 4 varieties. Line is best fit for all data points except shade.

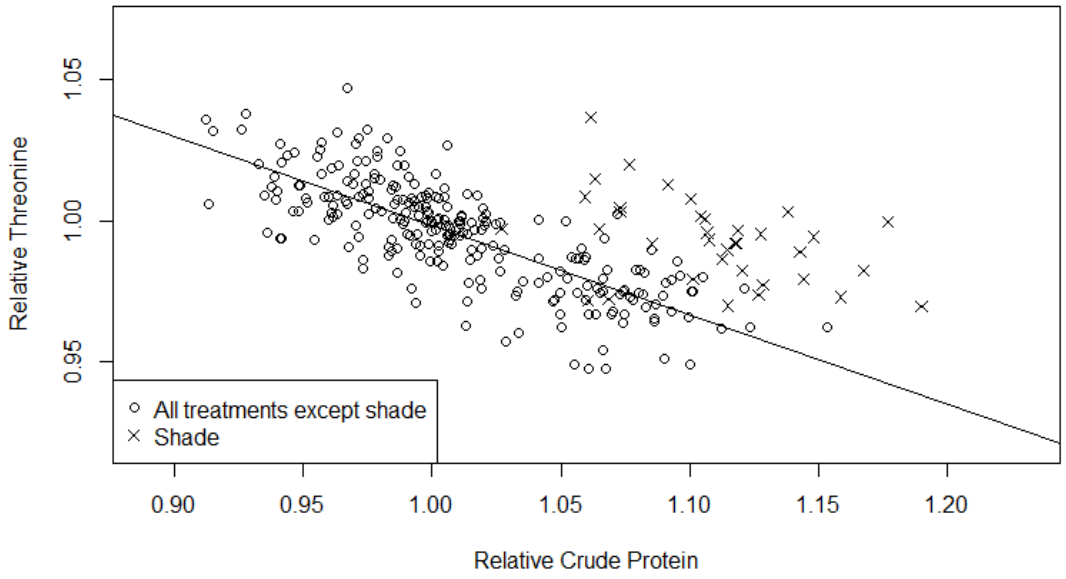

Figure 5. Relationship of relative threonine with relative crude protein across 5 environments and 4 varieties. Line is best fit for all data points except shade.

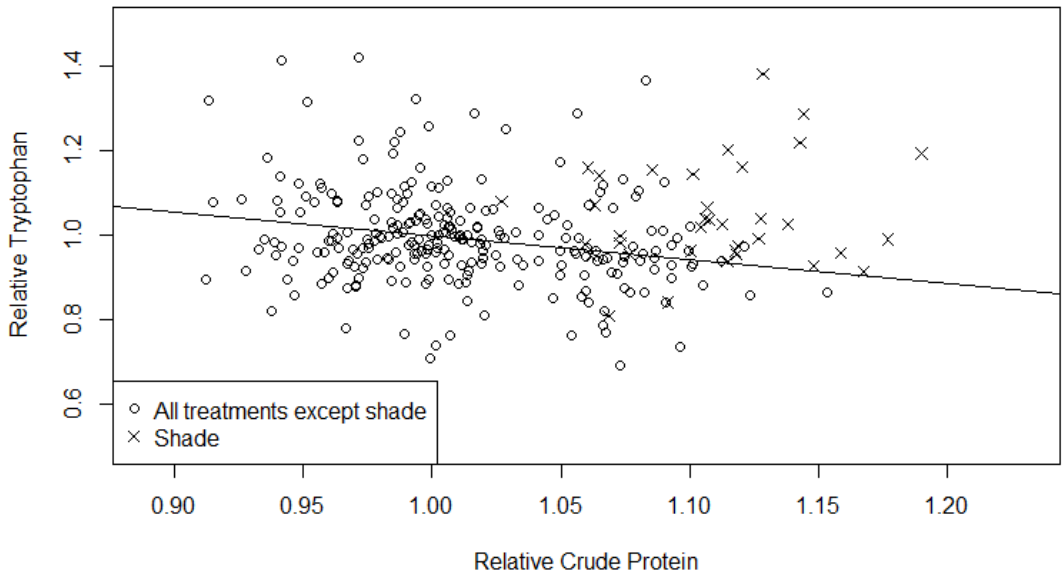

Figure 6. Relationship of relative tryptophan with relative crude protein across 5 environments and 4 varieties. Line is best fit for all data points except shade.

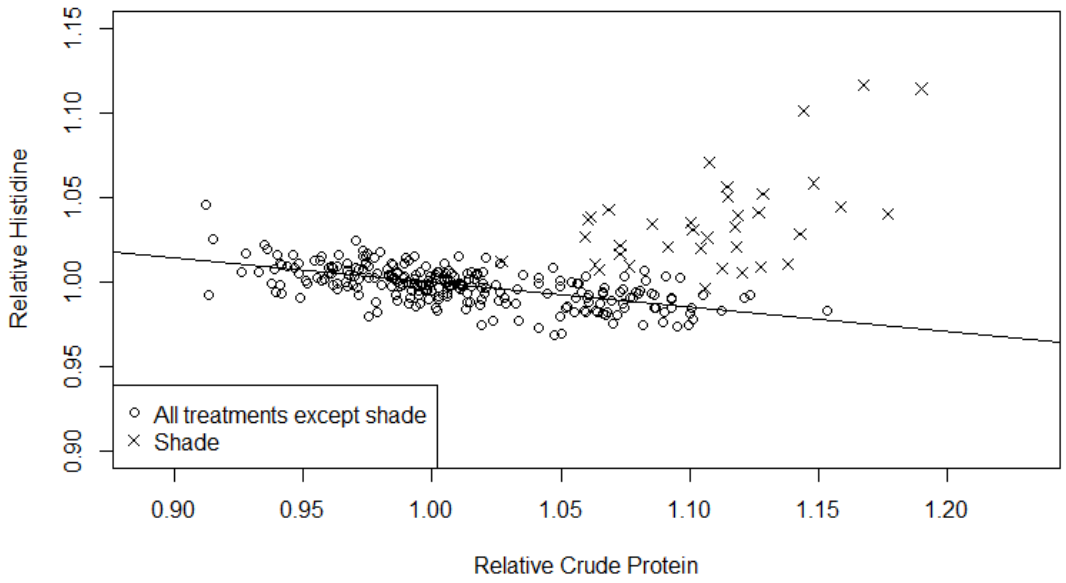

Figure 7. Relationship of relative histidine with relative crude protein across 5 environments and 4 varieties. Line is best fit for all data points except shade.

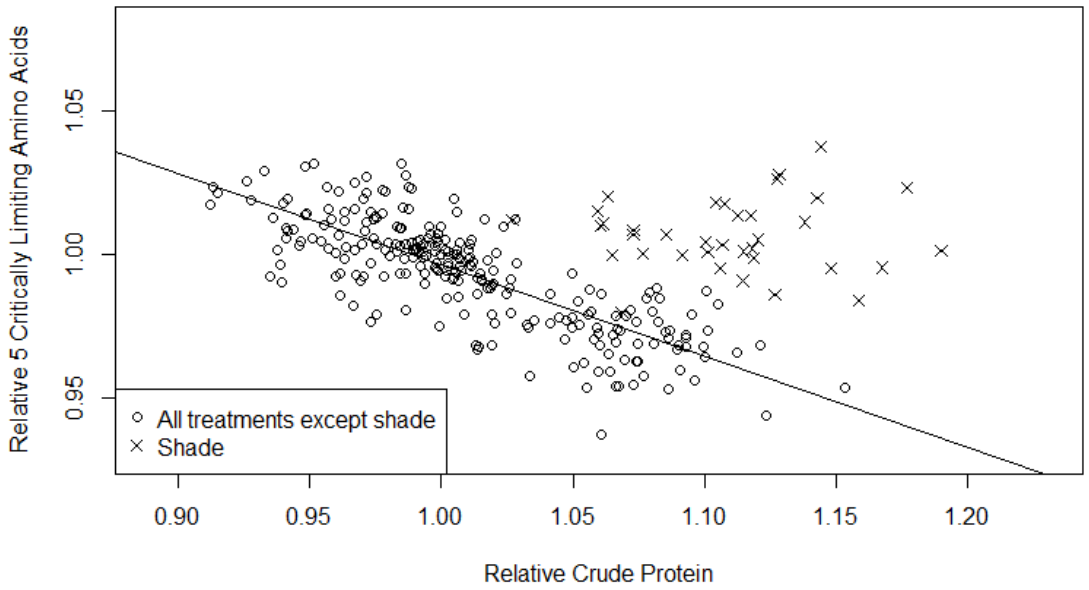

Figure 8. Relationship of relative sum of the 5 limiting amino acids with relative crude protein across 5 environments and 4 varieties. Line is best fit for all data points except shade.

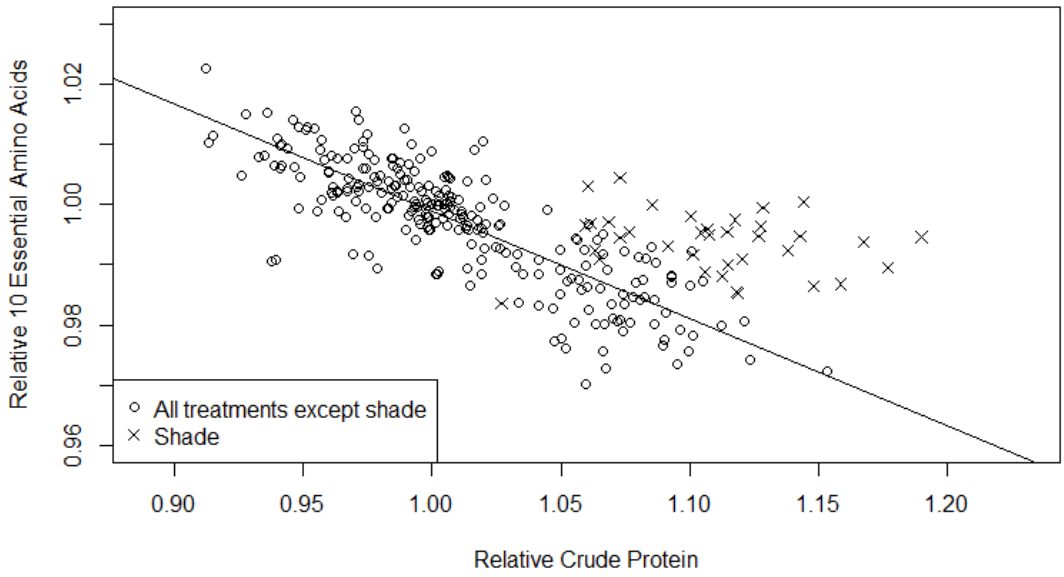

Figure 9. Relationship of relative sum of the 10 essential amino acidswith relative crude protein across 5 environments and 4 varieties. Line is best fit for all data points except shade.

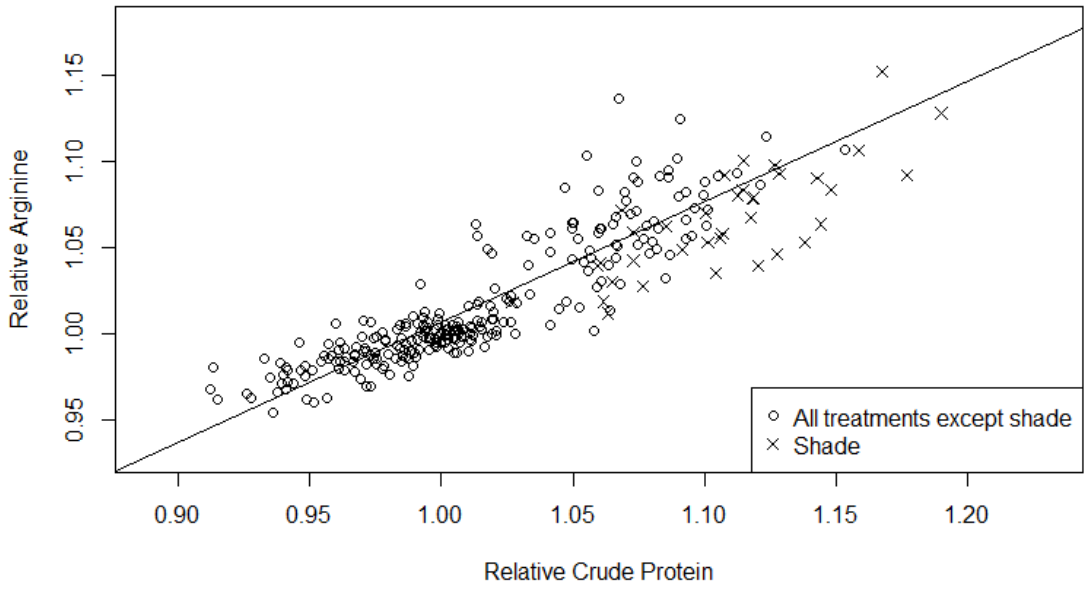

Figure 10. Relationship of relative arginine with relative crude protein across 5 environments and 4 varieties. Line is best fit for all data points except shade.

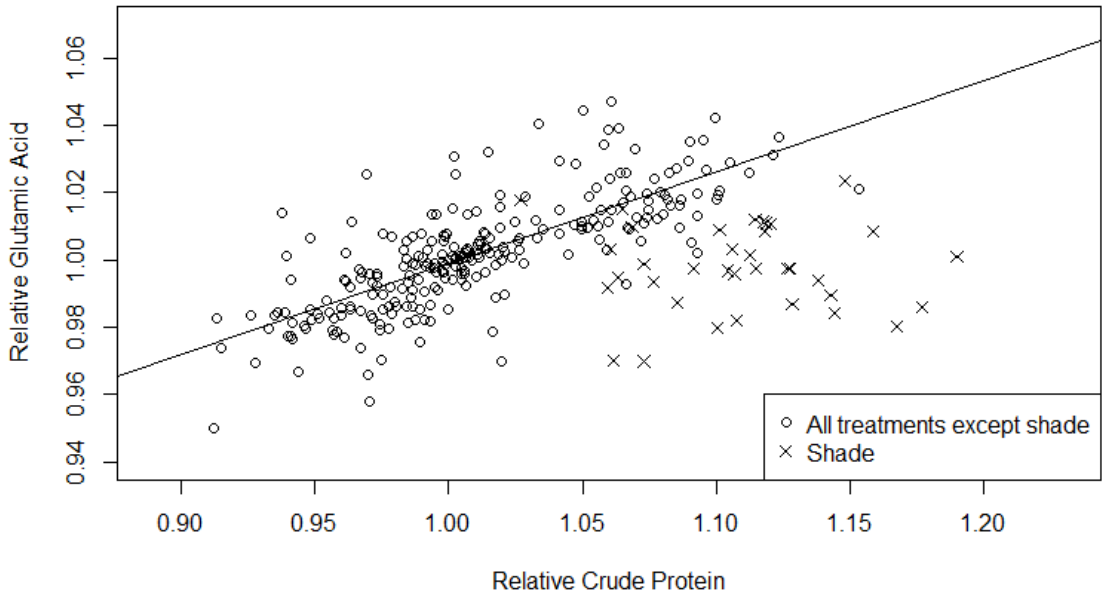

Figure 11. Relationship of relative glutamic acid with relative crude protein across 5 environments and 4 varieties. Line is best fit for all data points except shade.

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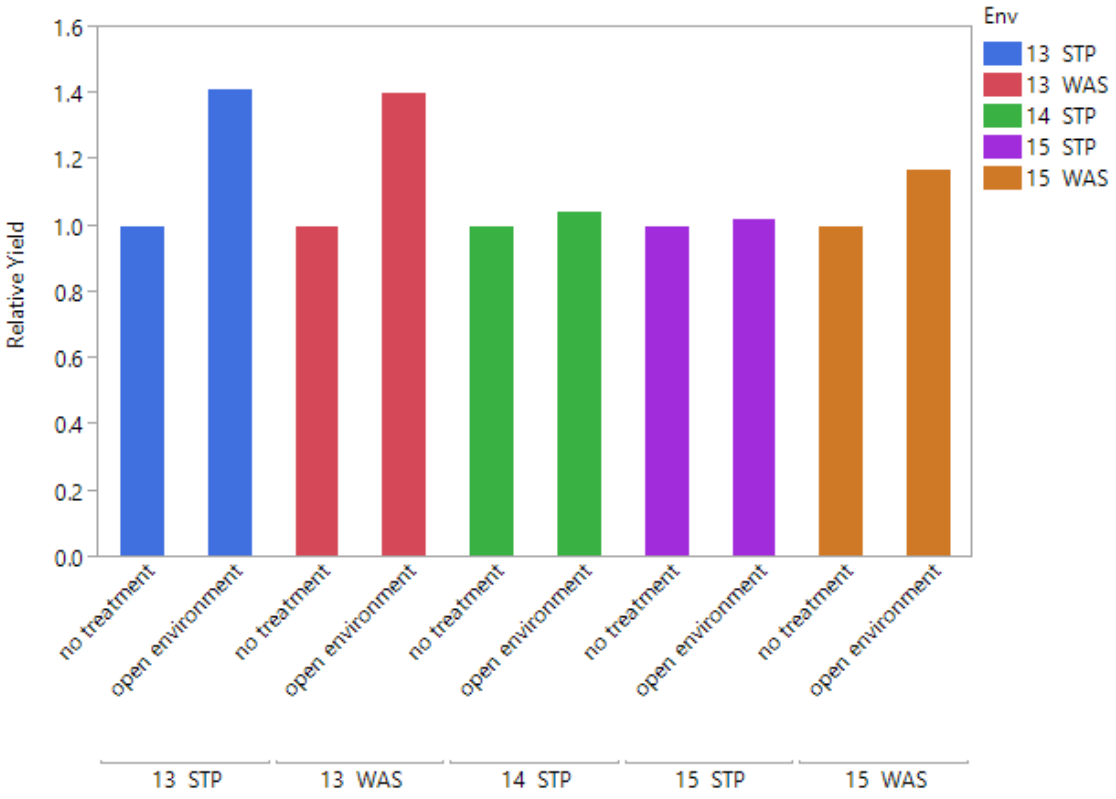
Appendix



Supplemental Figure. Relative yields are shown by no treatment (control) and open environment treatment to represent an environment by treatment interaction from the data set. Differences of magnitude, not direction, were observed between environments which was the source of environment x treatment interactions.

Treatment	Yield	Seed Number	Seed Size	Protein	Oil	P+O	Sum 18 Amino Acids
	Mg ha^{-1}	seeds m^{-2}	g 100 seed^{-1}	g kg^{-1} --			
Control	4.55 AB	2750 AB	16.8 C	339 C	179 A	518 B	327 C
Open environment	5.43 A	3030 A	18 B	345 C	182 A	527 A	330 C
Defoliation R5	3.35 CD	2310 BC	14.7 D	324 D	181 A	506 C	312 D
Defoliation R6	3.86 BC	2600 AB	15 D	326 D	183 A	509 C	314 D
Pod Removal 40%	2.51 DE	1270 DE	19.9 A	358 B	172 B	530 A	344 B
Pod Removal 70%	1.8 E	920 E	19.7 A	365 B	163 C	528 A	348 B
Shade 80%	2.03 E	1800 CD	11.6 E	376 A	149 D	524 AB	357 A
Multiple comparison	Mg ha^{-1}	seeds m^{-2}	g 100 seed^{-1}	g kg^{-1} --			
Dunnett's MSD (0.05)‡	0.82	480	0.9	6	4	6	7

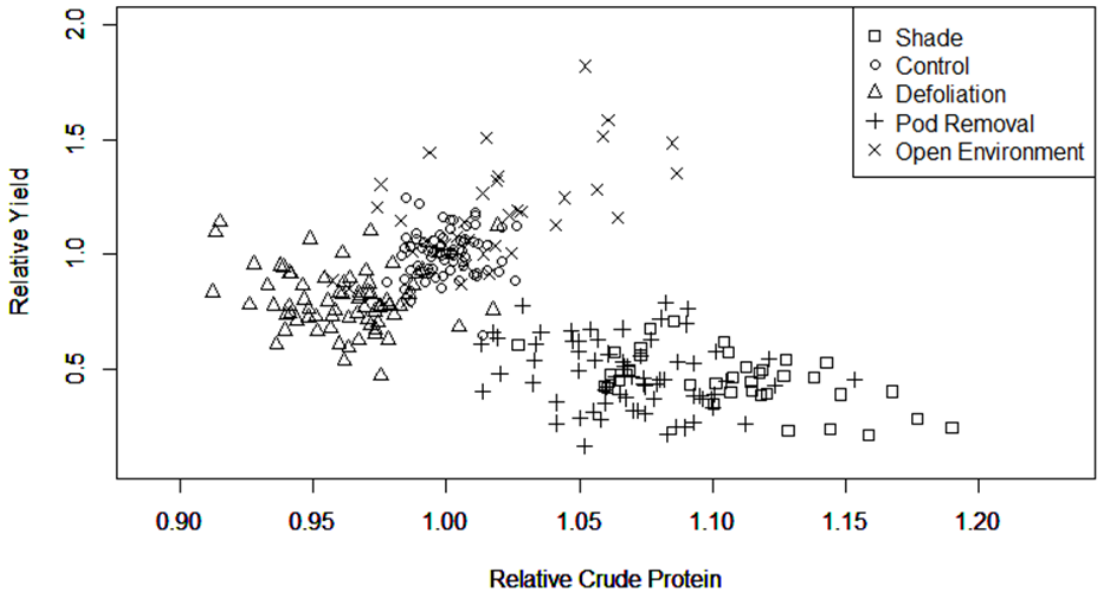
	AA group 1 – Oxaloacetate ‡						AA group 2-Ribose 5-phosphate
Treatment	Aspartic Acid	Isoleucine ¶	Lysine §	Methionine §	Threonine §	Total 1	Histidine ¶
Control	11.59 A	4.86 B	7.00 B	1.47 B	3.93 B	28.86 B	2.76 BCD
Open environment	11.58 A	4.87 AB	6.95 B	1.44 CD	3.90 B	28.74 C	2.74 CD
Defoliation R5	11.56 A	4.90 A	7.09 A	1.45 BC	3.98 A	28.98 A	2.78 B
Defoliation R6	11.56 A	4.88 AB	7.08 A	1.47 B	3.98 A	28.97 A	2.77 BC
Pod Removal 40%	11.59 A	4.81 C	6.88 C	1.42 D	3.85 C	28.55 D	2.73 CD
Pod Removal 70%	11.60 A	4.78 C	6.86 C	1.41 D	3.82 C	28.47 D	2.73 D
Shade 80%	11.61 A	4.73 D	6.98 B	1.50 A	3.91 B	28.72 C	2.86 A
Multiple comparison							
Dunnett's MSD (0.05)	0.04	0.03	0.05	0.02	0.04	0.09	0.03

Treatment	AA group 3 - Pyruvate				AA group 4 – Phosphoenol.+Ery.-4-phosphate			
	Alanine	Leucine ¶	Valine ¶	Total 3	Phenylalanine ¶	Tryptophan §	Tyrosine	Total 4
Control	4.09 B	7.92 AB	5.06 BC	17.08 B	5.42 AB	1.01 AB	3.74 C	10.18 AB
Open environment	4.07 BC	7.94 AB	5.06 ABC	17.08 B	5.45 A	1.02 AB	3.75 BC	10.23 A
Defoliation R5	4.16 A	7.97 A	5.10 A	17.23 A	5.43 AB	1.01 ABC	3.81 A	10.25 A
Defoliation R6	4.14 A	7.94 AB	5.07 AB	17.15 AB	5.42 AB	1.03 AB	3.79 AB	10.25 A
Pod Removal 40%	4.02 DE	7.88 BC	5.02 CD	16.93 C	5.44 A	0.97 BC	3.70 D	10.11 BC
Pod Removal 70%	4.00 E	7.85 C	4.99 DE	16.84 C	5.41 AB	0.95 C	3.67 D	10.03 C
Shade 80%	4.04 CD	7.73 D	4.96 E	16.73 D	5.39 B	1.05 A	3.68 D	10.12 BC
Multiple comparison		ns						
Dunnett's MSD (0.05)	0.02	0.06	0.03	0.08	0.04	0.05	0.04	0.08

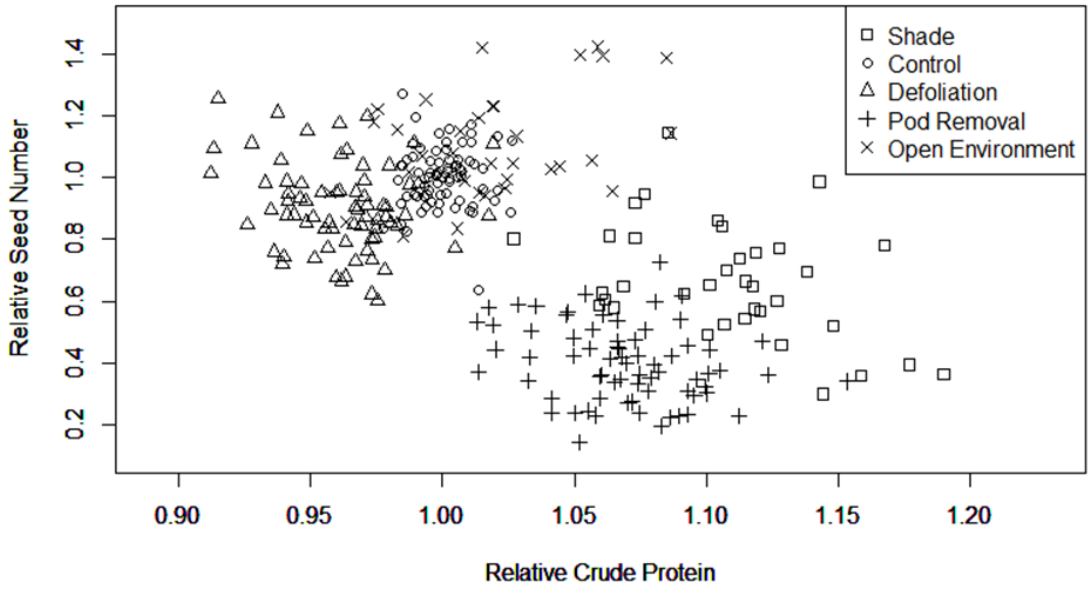
Treatment	AA group 5 - 3-Phosphoglycerate				AA group 6 - α-Ketoglutarate			
	Cysteine §	Glycine	Serine	Total 5	Arginine	Glutamic Acid	Proline	Total 6
Control	1.43 B	4.12 B	4.42 A	9.97 AB	7.70 CD	18.45 B	5.02 AB	31.17 C
Open environment	1.37 BC	4.09 BC	4.43 A	9.9 BC	7.79 C	18.53 B	5.00 AB	31.33 C
Defoliation R5	1.4 BC	4.17 A	4.44 A	10.01 A	7.56 E	18.19 C	5.00 AB	30.75 D
Defoliation R6	1.43 B	4.17 A	4.43 A	10.03 A	7.57 DE	18.23 C	5.02 AB	30.82 D
Pod Removal 40%	1.36 C	4.04 DE	4.38 A	9.79 D	8.10 B	18.74 A	5.06 A	31.90 A
Pod Removal 70%	1.35 C	4.03 E	4.44 A	9.81 CD	8.29 A	18.82 A	5.02 AB	32.12 A
Shade 80%	1.51 A	4.07 CD	4.41 A	9.99 AB	8.21 AB	18.41 B	4.97 B	31.58 B
Multiple comparison								
Dunnett's MSD (0.05)	0.06	0.03	0.07	0.07	0.11	0.14	0.05	0.19

Treatment		Sum 5 Limiting AAs §	Sum 10 Essential AAs ¶
	Control	14.85 AB	40.87 B
	Open environment	14.68 B	40.74 BC
	Defoliation R5	14.93 A	41.11 A
	Defoliation R6	15.00 A	41.08 A
	Pod Removal 40%	14.48 C	40.37 D
	Pod Removal 70%	14.39 C	40.14 E
	Shade 80%	14.95 A	40.61 C
Multiple comparison			
	Dunnett's MSD (0.05)	0.15	0.16

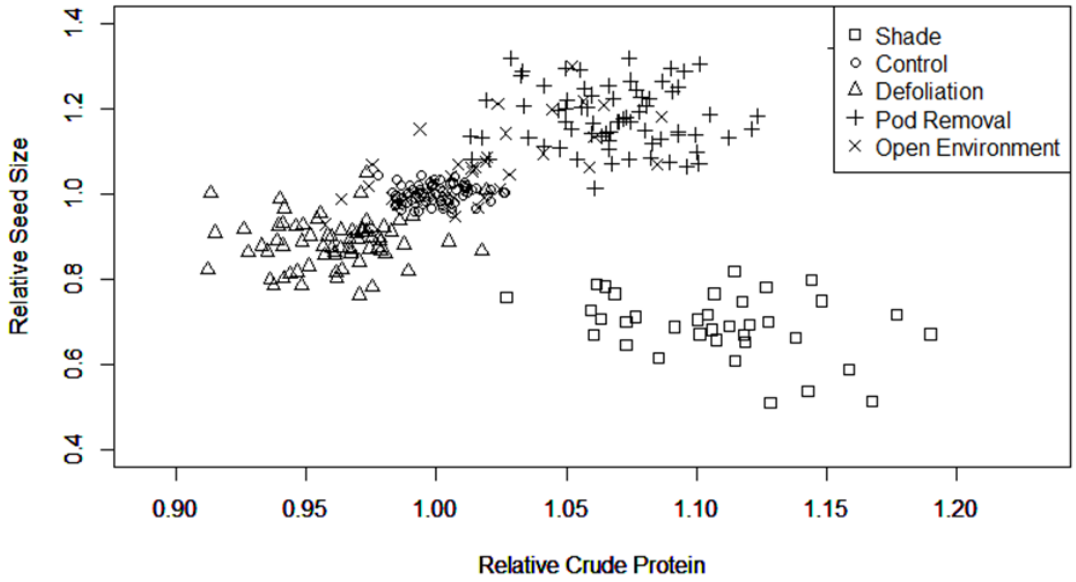
* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.
† ns, nonsignificant.
‡ Amino acids displayed as percent of 18 amino acids
§ The five limiting amino acids in soybean protein are lysine, methionine, cysteine, threonine, tryptophan
¶ The ten essential amino acids are lysine, methionine, cysteine, threonine, tryptophan, isoleucine, leucine, valine, histidine, phenylalanine



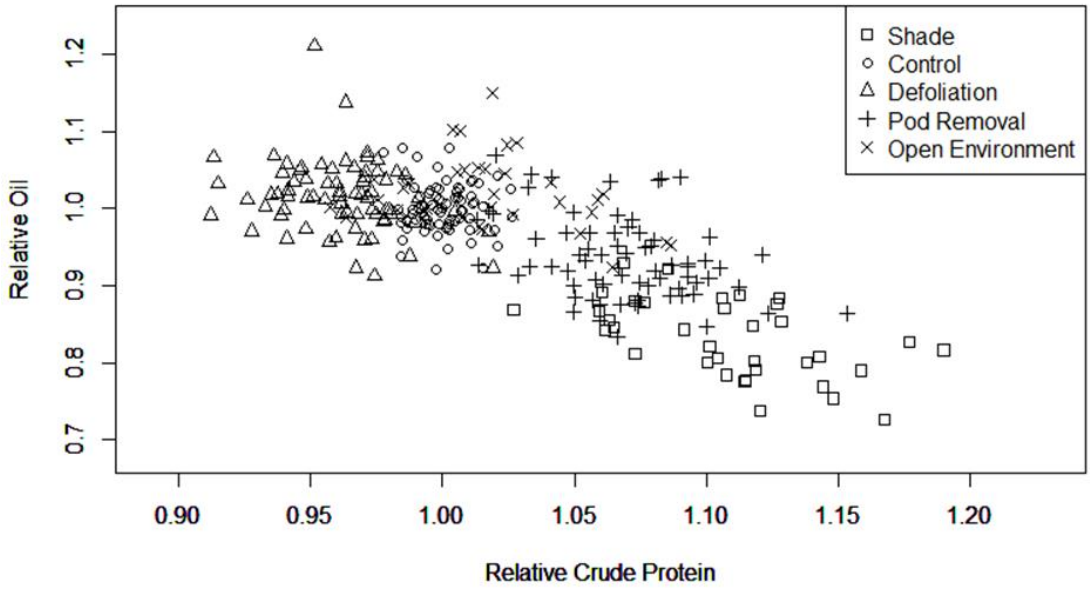
Relationship of relative yield with relative crude protein across 5 environments and 4 varieties. Data point symbol indicates treatment.



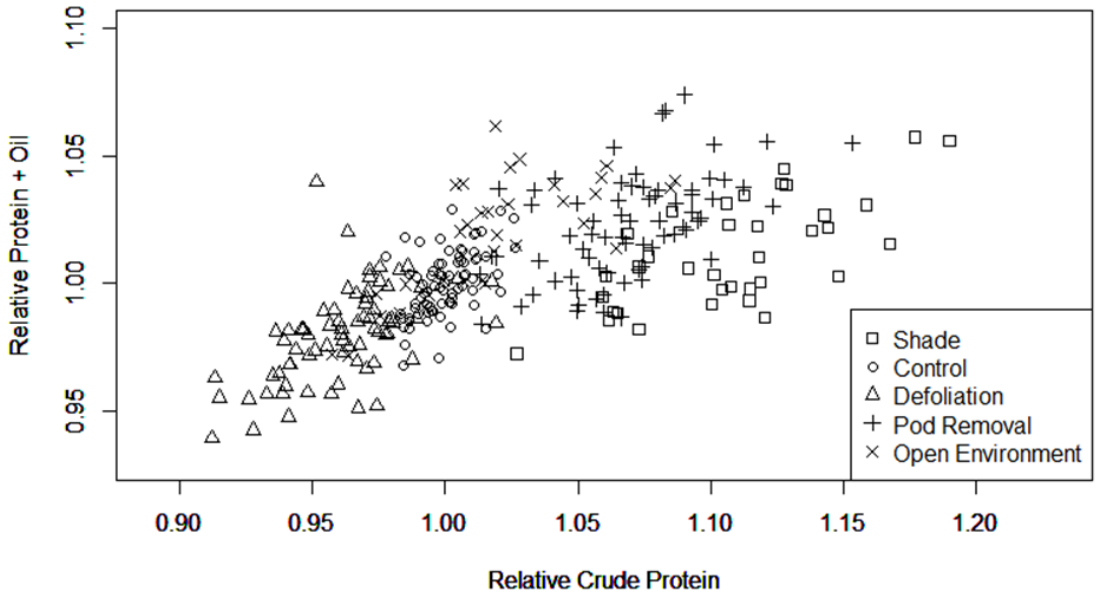
Relationship of relative seed number with relative crude protein across 5 environments and 4 varieties. Data point symbol indicates treatment.



Relationship of relative seed size with relative crude protein across 5 environments and 4 varieties. Data point symbol indicates treatment.



Relationship of relative oil with relative crude protein across 5 environments and 4 varieties. Data point symbol indicates treatment.



Relationship of relative crude protein plus crude oil with relative crude protein across 5 environments and 4 varieties. Data point symbol indicates treatment.