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Breeding for Modified Seed Composition in Soybean: Selection of Genotypes, Yield Stability, and Environmental Effects

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of the requirements for the degree of
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ABSTRACT

The development of cultivars with modified seed composition represents an excellent alternative to cope with the world's need for more nutritious food. Understanding the genetic and environmental factors controlling crop seed quality traits is of crucial importance for such an endeavor. The objectives of this study were: a) to evaluate the efficiency of phenotype and marker-assisted selection for low stachyose and low phytate soybean breeding lines, and their yield stability, b) to study the effect of management practices planting date and delayed harvest on soybean seed composition, and c) to study the potential association between soil properties and leaf chemical element concentration, with seed composition. Selection efficiency in six breeding populations segregating for the low phytate and low stachyose traits was studied by determining the proportion of phenotypically selected lines that carried the alleles for the low phytate trait or low stachyose traits. Yield stability of low phytate/low stachyose lines was studied in a set of 16 breeding lines selected from a breeding population segregating for the low phytate/low stachyose trait, and grown in five Arkansas environments. Planting date effects on seed composition were studied in nine breeding lines planted in early May, late May, and late June at two Arkansas locations in two consecutive years. Delayed harvest effects on seed composition and the association between soil properties and leaf chemical element concentration, with seed composition were studied in the late May planting date. Results showed that the efficiency of marker-assisted selection depended on the type of marker used, whereas phenotype-assisted selection depended on the germplasm that was being screened and on the phenotype used to make breeding selections. Breeders should use marker-assisted selection for low phytate/low stachyose lines only if phenotyping large number of progenies is not time or cost effective. Most of the low phytate/low stachyose lines showed low yield stability. However, one

of the low phytate/low stachyose lines studied, R08-6009, showed competitive yield and adaptation to all the environments where the line was evaluated. R08-6009 should be crossed to high-yield lines to generate progeny with greater yield potential. Other lines studied showed moderately high oleic (>45 mg g^{-1}) and low linolenic (<34 mg g^{-1}) acids concentrations in the oil, which are novel and unique combinations with the low phytate/low stachyose trait. Planting date significantly affected seed organic and inorganic composition. Early planting increased seed protein, oleic acid and decreased linolenic acid, whereas late planting increased sucrose, but did not affect stachyose. Production of soybeans with high protein/high quality oil should be performed in early-planting production systems, whereas planting high sugar food-grade cultivars late in the season should fit well in a double-crop system. Although seed components tended to decrease when harvest was delayed, the magnitude was not large enough to recommend to purposely change the time of harvest to attain a particular seed composition (e.g. low seed calcium). Attempting to modify composition by nutrient fertilization may not be profitable, as no association between leaf or soil chemical elements with seed composition was observed. The findings reported in this dissertation may help: 1) develop low phytate/low stachyose soybean breeding lines/cultivar adapted to Arkansas, 2) understand how those breeding lines can be developed in a shorter period of time (increase selection efficiency), 3) deliver specialty soybean cultivars that come not only come with improved seed quality traits and high yield potential, but also with supporting information on some management practices (planting date, time of harvest, response to specific soil nutrients) that may help farmers meet market specifications and contribute to sustainable agriculture.

This dissertation is approved for recommendation
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DEDICATION

I dedicate this dissertation to my wife, Silvana Dormi.

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I. Introduction

Soybean [(*Glycine max* (L.) Merr.] is an important oil seed crop produced throughout the world. The U.S. has 35% of the total world soybean production, followed by Brazil, Argentina, and China, which jointly account for approximately 52% (Soystats, 2011). Soybean production has been increasing steadily during the last three decades. In 2008, for example, the U.S. produced 81.6 millon metric tons (mmt) of soybeans, which was one of the highest in the last 5 years. A large portion of the crop is exported to other countries, with China and Mexico being the main consumers (Soystats, 2011). The bulk of the soybean crop is solvent extracted for edible oil and biofuel, and the defatted soymeal is used for animal feed (Kerley and Allee, 2003; Soystats, 2011). Soybean accounts for 68% of the protein meal (Golbitz, 2000; Soystats, 2011) and 56% of the world edible oil consumed. Although the major breeding target in soybean, as in other crops, has always been increasing yields, breeding efforts directed to changes in seed composition have recently gained importance. This has translated into breeding efforts towards soybeans with increased oil and protein concentration (Chen et al., 2008), and specific (e.g. low linolenic acid) fatty acid profiles in the oil (Fehr, 2007).

Dry soybean seed consists of about 40% protein, 20% oil, 35% carbohydrate, and 5% ash. The majority of soy protein is relatively heat-stable and includes three major groups defined by sedimentation value: 2S (α-conglycininss), 7S (β-conglycinins), and 11S (conglycinin). High protein concentration has been shown to be correlated with tofu yield and firmness (Poysa and Woodrow, 2002). Further, soybeans with high protein concentration produce soybean meal with increased nutritional value for soybean-based animal feed. Commercial soybean oil consists, on average, of: 10 milligrams per gram of oil (mg g-1) palmitic acid (16:0), 4 mg g^{-1} stearic acid (18:0), 22 mg g^{-1} oleic acid (18:1), 54 mg g^{-1} linoleic acid (18:2), and 10 mg g^{-1} linolenic acid

(18:3). The first number of the fatty acid abbreviations indicates the length of the fatty acid in number of carbon atoms, and the second number indicates the number of double carbon-carbon (unsaturated) bonds in the molecule (Wilson, 2004). High oleic acid and low linolenic acid make soybean oil healthier (higher concentration of monounsaturated fats, lower concentration of polyunsaturated fats), and improve oil oxidative and heat stability, which is more attractive for frying and edible applications. Low saturated fatty acids (stearic and palmitic acids) concentration also contributes to oil quality and flavor stability and may help reduce the risk of coronary disease (Simopoulos, 1999). The major soluble carbohydrates of mature soybeans are: sucrose [35-55 mg per gram of seed (mg g^{-1})], raffinose (11-16 mg g^{-1}) and stachyose (29-36 mg g^{-1}) (Liu, 1997; Górecki et al., 2001; Guillon and Champ, 2002; see also Karr-Lilienthal et al., 2005). High sucrose and low stachyose improve soyfood flavor and digestibility (Mebrahtu and Devine, 2009) and the nutritional value of animal feed. The rest of the seed consist of other carbohydrates, phytate, and inorganic chemical elements with Fe and Zn being the one in highest concentrations.

The incorporation of soybean meal in animal feed has raised concerns about its antinutritional components such as oligosaccharides and phytic acid. Sucrose plays a role in germination by providing energy to the growing seedling (Gardner et al., 2003). Oligosaccharides (raffinose and stachyose), however, may not be as important in the germination process (Dierking and Bilyeu, 2009), but they have been thought to be involved in seed desiccation resistance (Black et al., 1996; Obendorf, 1997), serve as transport carbohydrates in the phloem (McCaskill and Turgeon, 2007), and as storage reserves and cryoprotectants in frost-hardy plant organs in some plants (Pennycooke et al., 2003). Sucrose is fully digested by animals consuming soybean meal, and therefore, represents a valuable source of energy to the growing

livestock. Additionally, sucrose contributes to the sweetness of soybeans that are processed for making soyfoods (Abe et al., 2004). On the other hand, oligosaccharides are not broken down into simple monosaccharides by monogastric animals due to lack of the enzyme α-galactosidase in animals' digestive system (Górecki et al., 2001; Guillon and Champ, 2002; Karr-Lilienthal et al., 2005). Thus, oligosaccharides reduce the amount of metabolizable energy in monogastric animals that consume feed enriched with soybean (Coon et al., 1990), which represents an obstacle for an efficient utilization of soybean meal in animal feed (Sebastian et al., 2000) and production of healthy soyfoods for human consumption. Reducing the oligosaccharide concentration from soybean meal can increase its metabolizable energy as much as 20% (Sebastian et al., 2000; Dierking and Bilyeu, 2008). Breeding efforts towards decreasing the amount of oligosaccharides and increasing sucrose concentration in soybean seed represents a useful strategy in engineering highly metabolizable soybean cultivars.

A second well-recognized antinutritional component of soybean meal is phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate), or phytate, the phytic acid salt. Phytate is the form in which 67 to 77% of the phosphorus (P) in soybean seed is stored (Walker et al., 2006) and a molecule that chelates other essential inorganic elements. Phytate is the primary source of P and other elements for the germinating seed and the growing seedling (Gardner et al., 2003). Because P is chelated to the phytic acid skeleton and monogastric animals lack the enzyme phytase (Yamka et al., 2005), most of the P in the soybean seed is not available for absorption. Thus, a large portion of the P consumed by livestock is excreted. Land fertilization with manure derived from livestock holding facilities (e.g. chicken houses) can result in runoff and leaching of excessive P, and eutrophication of surface and ground water (Ertl et al., 1998). Identifying genetic sources of low phytate for major crops (Raboy, 2007; Raboy, 2009) and improving farm

waste and nutrient management (Kornegay and Harper, 1997) may be useful alternatives to cope with this problem.

The main goal of a plant breeder is to develop cultivars that have high yield potential and agronomic and seed quality traits that meet market standards, and give producers the most profit. Thus, developing this type of cultivar consists of combining traits that help improve yield potential, seed quality, and their stability. However, trait expression depends on the genotype, the environment where the genotype grows and develops, and on the effects caused by the interaction between the genotype and the environment (Lewontin, 2000). Therefore, in order to maximize the expression of traits of interest (e.g. seed protein concentration) it is necessary to a) find stable genetic sources for the trait and b) understand what environmental conditions (e.g. soil, air temperature, soil moisture) have a significant impact on the genotypes selected by the breeder. More importantly, it is necessary to understand, if possible, how to manipulate those effects in such way that they maximize crop yield and quality. Thus, in this literature review I will provide an overview of the genetic sources that are currently used to breed for soybeans with reduced antinutritional components (phytate, oligosaccharides), including their genetic base and environmental effects, and their correlation with other traits. In the remaining section I will review the literature on three components of the environment that can have effects on overall seed composition. The environmental components include two management practices, planting date and delayed harvest, and soil properties. The same order will be followed for the chapters presented in this dissertation.

Description, selection and yield potential of genetic sources of low phytate and low stachyose

A low stachyose genotype PI 200508 has been identified (Sebastian et al., 2000; Hitz et al., 2002) (Table 1). PI 200508 exhibits increased levels of sucrose (65 mg g^{-1}), reduced levels of raffinose (5 mg g^{-1}) and stachyose (15 mg g^{-1}), and normal levels of phytate (18 mg g^{-1}) (Gao et al., 2008; Saghai Maroof et al., 2009). PI 200508 showed up to 65% reduction in stachyose concentration when compared to the average of eight elite cultivars (Sebastian et al., 2000). The inheritance of the low stachyose phenotype followed a 3 (normal stachyose) to 1 (low stachyose) ratio, which suggested that one recessive allele (*stc1*) was responsible for the sugar profile of this genotype (Sebastian et al., 2000; Dierking and Bilyeu, 2008). PI 200508 carries a 3 bp (base pair) deletion in the gene raffinose synthase, which is involved in both raffinose and stachyose synthesis during seed development (Dierking and Bilyeu, 2008; Skoneczka et al., 2009). *Stc1* belongs to the galactosyltransferases group, which shares close homology with raffinose synthase genes from other plant species including pea (*Pisum sativum*), cucumber (*Cucumis sativus*) and *Arabidopsis* (Skoneczka et al., 2009). Individuals carrying this mutation can be distinguished with the simple sequence repeat (SSR) marker *Rsm-1* located on linkage group (LG) C2. The incorporation of low stachyose alleles from PI 200508 into elite cultivars has proven to be feasible and without major negative consequences on agronomic traits (Neus et al., 2005), or seed components such as protein concentration (Sebastian et al., 2000). Neus et. al. (2005) studied seed and agronomic traits of two single-cross populations between the lines LR01, a backcross-derived breeding line with 12.5% of PI 200508 genome, and two high-yield cultivars. The authors found no significant differences between the performance of lines with low stachyose and the performance of normal stachyose alleles for field emergence, seed yield, maturity, lodging, height, protein, oil, or fatty acids. PI 200508 is a natural mutant found in the

United States Department of Agriculture (USDA) seed repository and it is available to public breeders.

A low phytate/low stachyose line LR33 was developed by chemical mutation by Sebastian et al. (2000) (Table 1). LR33 exhibits low seed oligosaccharides and reduced phytate concentrations (Sebastian et al., 2000; Hitz et al., 2002). The reduction in oligosaccharide concentration may reach up to 90% of a normal cultivar. Similarly, the phytate concentration of LR33 decreased twofold as compared with wild-type lines (Hitz et al., 2002). A mutation in the enzyme D-*myo*-inositol 3-phosphate synthase 1 gene (*Mips*), which converts glucose 6-P, the substrate for the sugar synthesis pathway, into *myo*-inositol 6-P is responsible for the changes in seed phytate and oligosaccharides concentrations (Chapell et al., 2006). Because this mutation is early in the biosynthetic pathway, it leads to a decrease in both oligosaccharides and phytate. The reduction in oligosaccharides is greater in LR33 than in PI 200508. Further, the LR33 mutation causes a greater decrease in the amount of stachyose produced than it does for raffinose, whereas the PI 200508 mutation has the opposite effect (Sebastian et al., 2000; Hitz et al., 2002). LR33 showed low levels of germination and field emergence when first discovered. However, when the LR33 mutation was incorporated into other cultivars, several lines that carried the LR33 mutation had adequate germination and field emergence (Sebastian et al., 2000). LR33 is privately owned and it is not available to public breeding programs.

A low phytate/low stachyose line V99-5089 was developed by Virginia Polytechnic Institute and State University (Saghai Maroof and Buss, 2011) (Table 1). V99-5089 contains high sucrose (75-90 mg g^{-1}), low raffinose (5 mg g^{-1}), low stachyose (5 mg g^{-1}) (Florez-Palacios, 2009), and low phytate (9 mg mg^{-1}) (Gao et al., 2008; Saghai Maroof et al., 2009), caused by a single nucleotide recessive mutation in the *Mips* gene, which is different than the mutation in LR33.

The segregation of the low stachyose phenotype follows, as expected for a recessive gene, a 3 (normal stachyose) to 1 (low stachyose) ratio (Huhn, 2003). Recent work showed that a QTL linked to SSR marker Satt453 on LG B1 explained large percentage of the variance in stachyose concentration in a segregating population derived from the cross V99-5089 x 'Essex', and also contributed largely to the low phytate phenotype (Saghai Maroof and Buss, 2011). V99-0589 has been crossed to other lines in order to incorporate its unique traits into high-yield backgrounds (Maupin et al., 2011b) and combine them with other quality traits. Maupin and Rainey (2011) evaluated three V99-5089-derived low phytate lines at six environments and found that the average yield of the lines ranged from 3046 to 3250 kg ha^{-1}. Check cultivars 5601T and Essex had a yield of 3970 and 3495 kg ha^{-1}, respectively. Maupin and Rainey (2011) concluded that the mutation in V99-5089 had no negative impact on grain yield.

CX1834-1-2 is a low phytate breeding line derived from the cross between 'Althow', a commercial cultivar, and M153, a line developed by mutagenesis with ethyl methanesulfonate at Purdue University (Wilcox et al., 2000) (Table 1). M153 was also used to develop CX1834-1-3 and CX1834-1-6, two sister low phytate lines of CX1834-1-2. The three sister lines have been used in different studies and, to avoid ambiguities, they are all now referred as CX1834. The sugar concentration of CX1834 has not been published, but preliminary results from our lab showed that CX1834 exhibits normal sugar profiles with 35-45 mg g^{-1} sucrose, 8-11 mg g^{-1} raffinose, and 31-34 mg g^{-1} stachyose. The phytate concentration of CX1834 is lower than the one caused by the *Mips* mutation. Previous research showed that the low phytate phenotype in CX1834 is controlled by two recessive QTL (quantitative trait loci), mapped by Walker et al. (2006) and confirmed in Virginia (Gao et al., 2008) and Tennessee (Scaboo et al., 2009). These two QTL (*Lpa1*; *Lpa2*) are located on LG N and L, and are linked to SSR markers Satt237 and

Satt561, respectively. Recent work showed that the QTL on LG N contains a mutation in the multi-drug resistance-associated protein ATP binding cassette (MRP ABC) gene (Saghai Maroof et al., 2009). Further, Gillman et al. (2009) found that the two low phytate mutations in CX1834 are highly similar and possibly homologous genes. One of the major agronomic traits affecting low phytate lines derived from CX1834 lines is seedling emergence. Hulke et al. (2004) evaluated low and normal phytate lines derived from the cross of CX1834 and B01769B019, a reduced palmitic acid line. Low phytate lines had 22.3% lower emergence than the normal phytate lines. Similarly, Oltmans et al. (2005) developed three populations with CX1834 crossed to three different high-yield parents, IA 1008, IA 2050, and IA 2068. After selecting a subset of low and normal phytate lines, Oltmans et al. (2005) measured several agronomic and seed traits. The only significant difference between the low and normal phytate lines was observed in seedling emergence, with 68% emergence in normal phytate lines and 45% in the low phytate lines. Spear and Fehr (2007) incorporated the low phytate alleles from CX1834 into a commercial cultivar by successive backcrossing. The authors found 18 low phytate lines whose seedling emergence was significantly higher than CX1834 and not significantly different than the normal phytate parent. Recently, Trimble and Fehr (2009) crossed three of the low phytate lines developed by Spear and Fehr (2007), to the cultivars IA 2069 and IA 2070, to test whether additional backcrosses to normal phytate lines would improve the field emergence of low phytate lines. One of the crosses yielded low phytate lines with better emergence than the low phytate parent. Trimble and Fehr (2009) also pointed out that seed source was an important factor determining the success of identifying low phytate lines with good emergence. Gao et al. (2008) selected a subset of low and normal phytate lines from a population derived from the cross CX1834 x V99-3337 and grew them in two locations in Virginia. Unlike Oltmans et al. (2005)

and Hulke et al. (2004), Gao et al. (2008) did not find a consistent difference in seedling emergence across locations between the low and normal phytate lines. Seedling emergence appeared to be not only genetic, but also environment dependent for the population studied. The inheritance of seedling emergence in CX1834-dervied lines and the mechanisms behind its association with the low phytate trait are yet to be elucidated. All studies discussed above have agreed that the low phytate mutations do not have, assuming good emergence, a significant impact on grain yield.

Gm-lpa-TW-1 and *Gm-lpa*-ZC-2 are two low phytate lines developed recently by gamma radiation at Zhejiang University in China (Yuan et al., 2007) (Table 1). *Gm-lpa*-TW-1 and *Gm-lpa*-ZC-2 have a phytate reduction of 66.6% and 46.3% compared to their respective non-mutant parents. The two mutations are inherited as two non-allelic recessive genes. *Gm-lpa*-TW-1 mutation resulted from a 2 bp deletion in the *Mips* gene (Yuan et al., 2007) and it is different than the mutations in LR33 and V99-5089 (Maupin et al., 2011a). *Gm-lpa*-ZC-2 mutation was mapped on LG B2, closely linked to SSR markers Satt416 and Satt168, but the exact gene has not been characterized. *Gm-lpa*-TW-1 had consistently higher sucrose concentration and lower raffinose and stachyose concentration than its wild-type parents (Yuan et al., 2009). *Gm-lpa*-ZC-2 exhibited higher isoflavone concentration and the other seed components studied were not significantly affected. Frank et al. (2009a) reported lower inositol phosphates and increased *myo*-inositol concentration in *Gm-lpa*-ZC-2, which suggested that the mutation affects latter steps in the biosynthesis of phytic acid. *Gm-lpa*-TW-1 and *Gm-lpa*-ZC-2 mutations did not affect protein, amino acid, oil, saturated fatty acid concentrations (Yuan et al., 2009) or inorganic element composition (Frank et al., 2009b). Only *Gm-lpa*-TW-1 had reduced field emergence in unfavorable environments. *Gm-lpa*-TW-1 and *Gm-lpa*-ZC-2 have not been characterized in the

U.S. Studying their adaptation to soybean production areas and combining them with non-allelic mutations (*Lpa1, Lpa2, Rsm-1*) into a single line should be very valuable for breeders developing soybeans with low concentration of antinutritional seed components.

Marker assisted selection for low stachyose/low phytate lines

Several types of molecular markers have been used in identifying QTL affecting the expression of quantitative traits. Simple sequence repeat markers, one of the types of markers used in this study, consist of neutral repeating 1-4 base pair units that are not part of genes and, therefore, not translated to proteins. Simple sequence repeat markers have different alleles, which are given by the number of times the sequence is repeated, and therefore, the different size of the DNA fragment that is amplified by polymerase chain reaction (PCR). Single nucleotide polymorphism (SNP) markers, the second type of marker used in this study, help assess genetic differences at the nucleotide sequence level. Further, if the SNP is in the gene, recombination between the marker and the gene, unlike with SSR markers, is not a factor that may account for reduced selection efficiency. Thus, selection with markers in the gene (i.e “perfect” markers) markers would not produce false positives. The number of lines that have the alleles coding for a trait and are also phenotypically classified to have the trait will be a measure of phenotypic selection efficiency. Difference between these two group of individuals may only be attributed to variation caused by non-genetic effects.

A negative relationship between seed phytate and seed inorganic phosphorus (Pi) has been found for all genetic sources of low phytate. Because phytate chelates P, normal phytate seed contains low concentration of Pi, whereas low phytate mutants contain high concentration of Pi. Scientists have taken advantage of this association and used Pi to identify and select low phytate individuals carrying mutant alleles. Results from our laboratory at the University of Arkansas

showed that lines carrying the *Mips* mutation from V99-5089 had an average Pi about or higher than1000 µg g^{-1}, whereas lines carrying the two *Lpa* mutations had an average Pi about or higher than 2500 µg g^{-1}. These results may differ from those observed in other lines carrying the same mutations in different genetic background and/or grown in other soybean production areas.

Marker assisted selection efficiency for low phytate lines has been studied in CX1834 and V99-5089 germplasm. Scaboo et al. (2009) reported an overall (populations and marker combined) 50% marker-assisted selection efficiency using both SSR markers Satt237 and Satt561. Selection with these markers captured all high Pi individuals, but inheriting the CX1834 allele at both markers did not necessarily imply results in capturing a high Pi line. In contrast, Gao et al. (2008) found a complete association between phenotypic (phytate-based) and genotypic (SSR marker-based) selection in a breeding populations segregating for the *Lpa* genes. The authors of both studies suggested that, although a large percentage of the variance in seed phytate concentration was explained by the effects of *Lpa1* and *Lpa2* genes, other low phytate QTL were probably contributing to the trait. Maupin et al (2011a) studied a population of 153 recombinant inbred lines derived from the cross V99-5089 by Essex, a conventional high-yield line. Maupin et al. (2011a) reported 87% marker-assisted selection efficiency when using SSR marker Satt453 to select *Mips* mutants. Rosso et al. (2011) used the KBiosciences (Hoddesdon, UK) competitive allele specific PCR (KASPar) to genotype lines for the *Mips* mutation in a population of 142 F_2 lines derived from the cross V01-1693 x V03-5901. Results showed 100% efficiency to identify and classify mutant individuals. The KASpar system has not been used to genotype individuals in populations segregating for the low phytate genes *Lpa1* and *Lpa2*. Thus, incorporating the KASPar system for genotyping this germplasm should represent a valuable

asset for soybean breeders selecting low phytate individuals and would allow standardizing genotyping to a common platform independently of the source of the low phytate mutation.

Effect of management practices on seed composition: planting date

The effects of nutrient fertilization (Rengel et al., 1999; Yin and Vyn, 2003), water deficit stress (Dornbos and Mullen, 1992; Lee et al., 2008; Bellaloui et al., 2011), row spacing (Beatty et al., 1982), tillage (Temperly and Borges, 2006), shading and thinning (Izquierdo et al., 2009), and planting date (Kane et al., 1997; Ray et al., 2008; Bellaloui et al., 2011) on seed composition have been studied in soybean. However, planting date is probably one the factor that has, based on number of studies, received the most attention in the literature. Changes in planting date will expose the crop to a different environment (temperature, moisture, day length). For example, planting early in the season may expose seeds to wet and cool soils that may result in low germination and emergence, but may allow producers to harvest their crop before drought strikes during the seed-fill period. On the contrary, planting late may allow seedlings to emerge quick, but reproductive stages like ovule fertilization may take place in high temperature, producing significant flower abortion and consequently reduced yield. However, the seed-fill period may take place in a cooler environment than early planted soybean, which may lead to higher grain weight and quality.

One of the main obstacles towards fully understanding the effect of planting date on soybean seed composition has been the fact that most of the studies in this field cannot be compared among each other. Planting dates, planting date intervals, number of planting dates, type (bred for high yield vs bred for quality traits) of lines, maturity and number of cultivars used in the experiments may all vary considerably across studies. However, and after reviewing the literature, some general observations emerged (order does not imply importance) 1) late planted

soybeans experience cooler temperatures during the seed-fill period than early planted soybeans 2) early planting is usually correlated with and increase in oil and oleic acid and a decrease in sugars, linoleic and linolenic acids concentrations 3) saturated fatty acid showed the least response to changes in planting date or temperature of seed development 4) temperature during the seed-fill period is probably the most important environmental factor controlling seed organic composition 5) there are still some discrepancies on whether seed components are correlated to mean or high daily temperature 6) protein and oil responses to temperature during seed-fill period show a quadratic relationship 7) there are not many studies on the effects of planting date on seed sugar or inorganic element composition 8) results vary according to type of cultivar studied. In this review, I present literature supporting the eight overall conclusions mentioned above. I will include the most relevant papers to the topic and those that have been used as references by colleagues working in the same field of research. The bulk of the literature supporting statements above is derived from growth chamber studies, in which environmental factors were manipulated during plant growth, and field and long term data studies, in which seed composition was correlated with climatological variables.

Growth chamber studies

Sato and Ikeda (1979) studied the effect of increasing day/night temperature (17/12, 25/15, 25/20, 30/20, 30/25°C) on soybean seed organic and inorganic composition of the conventional (no quality traits) cultivar Miyagishirome. Oil, saturated fatty acid, oleic acid and iron (Fe) concentrations increased and total carbohydrates, linoleic, and linolenic acids concentrations decreased with temperature. In a similar study, Wolf et al. (1982) studied the effect of different temperature regimes (18/13, 24/19, 27/22, 30/25, 33/28°C) during soybean seed development on seed composition of the conventional cultivar Friskeby. Results showed that fatty acid

composition was strongly affected by temperature. Linoleic and linolenic acids decreased markedly, oleic acid increased with temperature and saturated fatty acids remained unchanged. Sucrose increased 56% with a 15°C decrease, stachyose responded with a slight reduction, and other sugars such as glucose, fructose and raffinose showed no response.

Dornbos and Mullen (1992) studied seed composition of potted soybean plants grown under different temperatures (28/20, 34/20°C) and with different levels of water deficit stress imposed during the seed-fill period. Severely water-stressed plants exhibited about 10.8% higher protein and 18% lower oil concentration than controls. The combined effects of high temperature and severe water deficit stress yielded seed with 14.8% more protein and 18.3% less oil than the controls. These results were conflicting with studies that had shown that high temperature would lead to lower protein concentration and higher oil concentration. Dornbos and Mullen (1992) proposed that the differences they observed were due to the significantly higher temperature they used in their experiments, as compared to previous studies. These results suggested that protein and oil responded in a quadratic fashion to temperature during seed-fill, showing opposite trends before and after a maximum/minimum (see Piper and Boote, 1999). Water deficit stress also affected fatty acid composition, but results were not consistent. Stressed plants had higher stearic acid and decreased oleic acid concentrations. Temperature of seed development decreased linoleic and linolenic acid (11.7% decrease of both acids combined) and increased oleic acid (10.7%). Gibson and Mullen (1996) studied the effect of temperature (30/20, 30/30, 35/20, 35/30°C) during different growth stages (R1-R8 = entire reproductive cycle, R1-R5 = flowering and pod set, R5-R8 = seed fill and maturation) on seed composition of the cultivar Gnome85. Changes in temperature during the flowering and pod set period had no significant effect on seed components. However, when temperature increased from 30/20 to 35/30°C during seed

development, protein and oleic acid concentrations increased, whereas oil, linoleic and linolenic acids concentrations decreased. In a different study, Gibson and Mullen (2001) studied the effect of temperature on seed inorganic composition. Calcium (Ca), manganese (Mn) increased and sodium (Na) concentrations decreased with higher temperature during flowering and pod set. Phosphorus, potassium (K), Ca, and magnesium (Mg) concentrations increased with higher temperature during the seed-fill period. Several inorganic elements increased with temperature when plants were exposed to different temperature regimes during the entire reproductive period.

Ren et al. (2009) studied the effects of high temperature imposed on the seed-fill and maturation period on seed composition of the high oleic acid breeding line N98-4445A. Fatty acid profiles and oil were significantly different between plants exposed to high temperature (37/30°C) and the control (27/18°C), whereas protein, sugars and phytic acid did not show a significant difference. High day and night temperatures increased saturated fatty acids, oleic acid and oil, but decreased linolenic and linolenic acids concentrations.

Field studies

Feaster (1949) studied yield and seed composition of five cultivars planted at 20 day intervals from April 20th to July 10th in southeast Missouri for four years. The effect of planting date on oil concentration depended on the cultivar. The oil concentration of three of the five cultivars was lower in the last planting date than the average of the first four planting dates. July planting resulted in the lowest oil concentration for all cultivars. The effect of planting date on protein concentration was opposite to the one observed for oil. Late planting date resulted in slightly higher protein concentration, but responses also depended on the cultivar being evaluated. In a similar experiment Weiss (1952) studied five cultivars planted on May 1st and four additional successive planting dates at 11 day intervals in three locations in the U.S. soybean belt for three

consecutive years. Weiss (1952) found that planting dates that resulted in higher mean daily temperature during the seed formation period increased seed oil concentration significantly. However, the association depended on the maturity of the cultivar studied. Like in Feaster (1949), protein concentration response was opposite to that of oil.

Wilcox and Cavins (1992) studied the effect of four plantings dates on oil composition of normal and low linolenic soybeans grown in one location in Indiana during five consecutive years. The first planting date was, on average, the second week of May, followed by four additional plantings separated by 7-10 days. The effect of planting date varied with years. Palmitic acid concentration increased slightly and stearic acid concentration decreased at later planting dates. Linolenic acid concentration increased with late planting dates, but the changes were not large enough to affect planting strategies of low linolenic soybeans. Regression analysis showed that an increase of 1°C in average maximum daily temperature during 20 days prior to maturity resulted in an average of 3.9 mg g^{-1} decrease in linolenic acid concentration.

Schenbly and Fehr (1993) studied the effect of planting date on the fatty acid composition of 10 lines with modified fatty acid profiles and two common cultivars at one location in Iowa during three consecutive years. Planting dates were the same (May 2nd, May 16th, May 30th, June 13th) for the three years (1988, 1989, 1990) of the study. The lines with elevated palmitic or stearic acid concentrations were not affected significantly by planting date. Early planting dates were associated with lower linolenic acid concentrations, but the difference between the earliest and the latest planting was only 4 mg g^{-1}. Unlike in Wilcox and Cavins (1992), average daily high temperature did not explain changes in linolenic acid concentration consistently. In a similar study in the southern U.S., Kane et al. (1997) studied soybean cultivars from maturity group 00 through IV planted in four planting dates. Kane et al. (1997) observed significant effect of

planting dates on seed fatty acid concentration. Late planting date was associated with increased protein and linolenic acid, and reduced oil and oleic acid concentrations. Planting date had little or no effect on saturated or linoleic acids. Higher temperatures experienced by early planted soybeans were strongly correlated with high oil and oleic acid, and reduced linolenic acid concentrations (Kane et al., 1997).

More recently, Oliva et al. (2006) studied the relationship between soybean seed fatty acid composition and average daily temperature of last 30 days before maturity of 13 soybean breeding lines differing in their fatty acid profile, and four check cultivars in 10 environments (two planting dates at five locations). Results showed that high oleic breeding lines were less stable than lines with normal oleic acid. Further, high oleic lines differed in oleic acid stability among them. Oleic acid and linolenic acid concentrations increased from 5 to 32.8 mg g^{-1} and decreased from 0.2 to 5.3 mg g^{-1} per °C increase in mean temperature, respectively. Bachlava and Cardinal (2009) found similar results to Oliva et al. (2006) in three breeding populations segregating for the high oleic trait grown in three locations in North Carolina for two consecutive years. Ray et al. (2008) studied eight breeding lines in which the low palmitic acid (five lines), low palmitic combined with low linolenic (two lines), and low linolenic (one line) traits had been incorporated by backcross. Breeding lines were planted early and late in the season at one location in South Carolina in three consecutive years. Early planting resulted in lower linolenic acid, higher protein and oil concentrations, whereas late planting date decreased palmitic acid concentration. Stearic and linoleic acids showed no significant differences between planting dates. Temperature during the seed-fill period was correlated with changes in fatty acid composition. Bellaloui et al. (2011) studied the seed composition of a single cultivar planted early and late in the season at one location during two consecutive years. Early planting

increased oil and oleic acid, but decreased protein, linoleic acid, and linolenic acid concentrations. Late planting was associated with an increase in sucrose and raffinose and a reduction in stachyose concentrations. Bellaloui et al. (2011) also found that late-planted soybeans accumulated more boron (B), P, and Fe in their seeds than those planted early. Except for Bellaloui et al. (2011), there have not been other studies on the effect of planting date on soybean seed inorganic composition.

Multi-year, multi-location studies

Piper and Boote (1999) performed analysis of the association between mean daily temperature during seed-fill period and seed protein and oil concentrations. The study included data of 20 conventional (no quality trait) cultivars representing 10 maturity groups (latitude 29.4 to 47.5° N) for a total of 1863 cultivar by location by year observations. A quadratic model was the best fit for the relationship between temperature during the seed-fill period and both protein and oil concentrations. Oil increased with temperature and reached a maximum at 28°C. Protein concentration decreased with temperature, but only a small portion of the total variance was explained by this factor. Photoperiod or water stress may have accounted for the pattern observed. In a similar and more recent study, Yaklich and Vinyard (2004) studied the relationship between seed protein and oil, and temperature and precipitation using data of USDA soybean collaborative yield trials from 1975 to 1983. Temperature during the month of September was the most influential on seed protein and oil concentration of all temperatures analyzed. Further, oil was also influenced by the sum of September minimum daily temperatures, whereas protein concentration was influenced by maximum daily temperatures. September is the month of the growing season when most soybeans, more or less depending on maturity, go through the seed development period. The two latter studies strongly support the hypothesis that

environment temperature during the seed-fill period is the most important non-genetic factor controlling organic seed composition.

Effect of management practices on seed composition: delayed harvest

Timely harvest is critical for obtaining maximum seed yield and quality. However, producers are sometimes faced with circumstances that do not allow harvesting the crop as soon as it reaches maturity and adequate moisture content. Unpredictable weather conditions (e.g. fall rains), unavailability of labor or machinery, or even time conflicts with other farm activities may lead to harvest delays. Moreover, some producers may delay the harvest (e.g. corn) on purpose in order to reduce grain drying costs (Thomison et al., 2011). It is important therefore to investigate and quantify the effects (if any) of delaying harvest in order to determine if they can cause a significant reduction in profit to the producer. Further, understanding the magnitude of these potential effects may help producers plan and prioritize their activities during the busy harvest season. Delayed harvest effects on seed composition are of significant importance for seed quality, especially for specialty crops, which must meet strict quality standard for the producers to maximize their investment.

The effects of delayed harvest on soybean seed composition have rarely been investigated. Most of the literature on the effects of delayed harvest focused on seed deterioration caused by disease (Wilcox et al., 1974; TeKrony et al., 1984), mechanical damage (Philbrook and Oplinger, 1989; Dao and Ram, 1996), and seed shattering (Philbrook and Oplinger, 1989). Krober and Collins (1948) compared the seed composition between good quality and weathered damaged soybeans. Delayed harvest and exposure to weather caused an increase in crude protein, decrease in sugars, and no significant effect on oil concentrations. Yaklich (1985) studied the effects of delayed harvest on seed sugars (sucrose, raffinose, stachyose) in six cultivars grown in Maryland

during three consecutive years. Seed of these cultivars was harvested at maturity and again after about six weeks. Delayed harvest had no significant effect seed sugar concentration. Further, the difference between soybeans harvested at maturity and those with delayed harvest were very small. For example, cultivar Miles had sucrose concentration at maturity = 73 mg g^{-1} and after the harvest delay = 74.3 mg g^{-1}.

In corn (*Zea mays*), Cloninger et al. (1975) studied the effects of delayed harvest on four hybrids at three plant densities and harvested at three dates (October 1st, November 1st, December 1st) at Columbia, MO for three years. Oil concentration declined 2.4% from the first to the second harvest delay, whereas protein concentration did not exhibit a significant response. The authors mentioned that the observed decline in oil was not large enough to be a concern for grain milling.

In cotton (*Gossypium hirsutum*), Christiansen and Justus (1963) studied seed deterioration in two cultivars grown in Stoneville, MS and harvested at three monthly intervals during November, December and January of the 1961 and 1962 seasons. The main finding in this study was that weather affected seed germination and increased the concentration of free fatty acids in the oil, a measure of oil deterioration caused by hydrolysis. Woodstock (1985) studied the effects of weathering on seed inorganic composition and traits related to vigor and germination in six cotton cultivars grown in Stoneville, MS in 1980. The first harvest was when the crop was mature, but before any significant weather event. The second harvest was five weeks later, a period in which seed had gone through periods of rain and temperature fluctuation. For one cultivar (Coker 201), seed that was harvested late showed slightly significantly higher K and Mg concentrations than seed harvested at maturity. The other elements studied, Ca, Na, and Mg, did not show significant differences.

In chickpeas (*Cicer arietinum*), Adak et al. (2007) harvested seed at three different times: before maturity (early), at maturity a week later (optimum), and a week after maturity (late). The experiment was conducted with a single cultivar at two locations in Turkey during one year. Protein, P, Ca, Mg, Co, Zinc (Zn) and Mn were higher at optimum harvest time than at either early or late dates. The authors proposed that the reason for the observed differences was that the time elapsed among harvest dates allowed for translocation of nutrients between vegetative and reproductive (seeds) parts of the plants (Adak et al., 2007).

Unlike organic seed components, seed inorganic elements do not undergo degradation and are not lost to cellular respiration as may happen to organic seed components (e.g. carbohydrates). Therefore, significant changes in inorganic composition after maturity (no element translocation) may not be expected. However, studies showed that when heavily weathered seed were incubated in pure water, inorganic elements were released in greater amount and faster than by seed that had been stored in dry and cool conditions. The differences observed in these experiments (Duke et al., 1983) indicated that elements were being lost from the seed, and that adverse conditions experienced by the seeds had damaged their cells and allowed nutrients to leak out into the medium (cell membrane leakage). Although measurements have not been taken under field conditions, it may be hypothesized that repeated cycles of high and low moisture (rain, sunny days) may lead to leakage of inorganic seed components and diluted in water present in the pod.

Effects of soil properties on seed composition

Because the soil is the source of chemical elements for plants, changes in soil mineral concentration may have the potential to affect a crop's mineral concentration. Therefore, modification of plant available element concentration in the soil may help modify seed inorganic

element concentration, which ultimately may be valued by markets focused on the production of high quality end-products derived from seeds. These effects may not be only on the seed chemical element concentration, but also may affect enzymatic reactions involved in the synthesis and translocation of organic components of interests (e.g. linolenic acid, protein). There are only few reports on how soil mineral concentration correlates with soybean seed inorganic element concentration. Identifying which seed elements may be significantly affected by soil properties and inorganic element concentration may be a starting point to develop fertilization practices that help producers attain seed market standards. This approach has been taken with other economically important traits like yield (Jiang and Thelen, 2004; Kumhálová et al., 2008) in several crops (Kravchenko and Bullock, 2000; Kravchenko et al., 2005; Ping et al., 2008; Basso et al., 2009).

Studies have been performed in order to understand fertilization strategies for improving plant health and maximizing yield. Only a small portion of those studies has been dedicated to changes in seed composition. Even less attention has been directed to physical soil features such as soil texture, slope and drainage. The main reason in explaining these differences are probably the little response that a researcher may expect if they were to set up such a study. However, in order to maximize production and quality of the crop one must look for and take advantage of new practices that, although they may not be significant at experimental levels, they can certainly, sometimes, have a great contribution at larger scales.

Soil chemical properties effects on soybean seed composition

Boswell and Worthington (1971) studied the effects of varying amounts of B and Mn on seed protein, oil, and fatty acids in a field experiment at three locations in Georgia using the soybean cultivar Bragg. Manganese and B had only a slight effect on protein concentration, but

differences were not significant. More recently, Bellaloui et al. (2009a; 2010) reported a significant increase in protein and oleic acid, but a decrease in oil and linolenic acid concentrations, when soybeans were foliar fertilized with B. Wilson (1982) reported that deficient levels of Mn increased seed linoleic, linolenic, saturated fatty acids and protein, but decreased oil and oleic acid concentrations. Ham et al. (1975) studied the effect of sulfur (S) fertilizer on seed protein and fatty acids and found no significant effect of S on these two seed components. Similar results on the effects of S fertilizer on protein and oil were observed in experiments performed in the soybean production states of Missouri (Brown et al., 1981), Kansas (Sweeney and Granade, 1993) and Iowa (Haq and Mallarino, 2005). Further, increasing levels of macronutrients N, P, K, Ca, and Mg in soybeans grown in hydroponic conditions did not affect seed composition (Harper, 1971).

Gaudou and Arrivets (1983) studied the effect of P, K, and N on soybean seed organic components and found that P increased protein and oleic acid, whereas K increased oil and linolenic acid, but decreased protein concentration. Sale and Campbell (1986) studied the effects of different levels of K in the growing medium of potted plants on organic seed composition. Plants growing in K deficient levels showed a decrease in oil and K concentration, but an increase in protein concentration. The rate of accumulation of oil and carbohydrates declined in K deficient plants compared to plants grown in optimum levels of K. Protein, on the other hand, showed no difference. Plants transferred to K-deficient medium during the seed-fill period showed no difference in seed composition, which indicated that K had been remobilized from vegetative tissue and its role on phloem translocation and protein synthesis had not been significantly affected. Further, resupplying K to plants that had been grown in K-deficient medium before anthesis did not have significant effect on seed composition. These results

showed that effects of K on seed composition will be observed only if plants are grown under deficient levels (see also Fernandez et al., 2009). Bellaloui et al. (2009b) found a significant positive correlation between soil K, B, and Zn and protein and oleic acid, but a negative correlation with oil, which at least for K, disagreed with Gaudou and Arrivets (1983), but confirmed the results observed by Sale and Campbell (1986).

Although some of the studies reviewed in this section showed that minerals in the soil may directly and/or indirectly affect the partition of organic compounds into the major seed components, it seems that large effects of soil mineral concentration on seed composition is more of an exception than a rule, as most of the studies have found little or no significant effects.

Soil physical properties effects on soybean seed composition

Kravchenko and Bullok (2000) studied the influence of field elevation, slope and curvature on seed protein and oil concentration in several fields in Illinois. The three variables were positively correlated with protein concentration in most of the fields studied. Oil was less affected by topography than protein concentration. The effects of topography interacted with weather patterns, which are known to play an important role in crop growth and seed composition. Martin et al. (2007) studied the relationship between soil properties (elevation, slope, electrical conductivity, soil reflectance, and soil organic matter) and vegetation indices, and soybean protein, oil and yield, in order to use remote sensing late in the season to harvest areas differentially. The study was conducted in two 16 ha field subsections in eastern Illinois for two years. The analysis revealed that vegetation indices (plant greeness and biomass) were positively correlated with protein, negatively correlated with oil, and were better predictors of these seed components than soil properties variables. In a similar study in corn, Miao et al. (2006) reported that after hybrid type, cation exchange capacity of the soil, Zn concentration of

the soil and aspect (line of steepest descent usually in degrees starting clockwise from the north) were the factors that most contributed to yield and protein concentration. Cation exchange capacity affects mineral nutrient availability and may indirectly affect overall plant growth and yield. Zn is important in activating many enzymes, including those involved in DNA replication and protein synthesis. Aspect affected solar radiation, evapotranspiration and soil water concentration, which are variables that influence the ability of the plant to produce organic matter, grow and uptake and transport nutrients in the transpiration stream. Other variables studied by Miao et al. (2006) that are important for plant functions also contributed, in a lesser degree, to yield and protein variation.

In wheat (*Triticum aestivum*), Stewart et al. (2002) studied the association between the soil variables pH, soil texture, electrical conductivity, field capacity, permanent wilting point, and yield and protein concentration in durum wheat grown in southern Australia. Areas with lower available water caused by coarser soil texture contributed to water stress during the seed-fill period, which interacted with soil nitrogen (N) and increased protein concentration. Norouzi et al. (2010) also found that N explained the largest portion of the grain protein in durum wheat grown in Iran, but other variables such as curvature, organic matter, available K, slope, and clay concentration also played a role. Fietz et al. (1994) evaluated the combined effects of N fertilization and landscape (footslope, south-backslope, shoulder, north-backslope) on yield and grain protein. Data analysis revealed that N fertilizer amount was correlated with grain protein and that protein tended to be lowest in north-backslope and greatest in the shoulder areas of the fields.

The studies described in this section have helped identify sources of variation affecting the crop and consequently contributed to the development of management strategies to efficiently grow high quality crops.

Hypotheses

1) Phenotype and marker-assisted selection will differ in their efficiency at selecting high Pi/low stachyose genotypes, and the efficiency of marker-assisted selection will depend on the genetic background in which the high Pi/low phytate mutations are expressed.

2) Planting date and harvest time will affect the organic and inorganic composition of soybeans seed, as the crop will experience different environments (e. g. temperature, moisture) at different planting dates.

3) Soil variables may have a significant effect on seed composition, as they greatly contribute to plant nutrient availability their mobilization and overall plant growth.

Objectives

The objectives of this study were to: 1) determine phenotype/marker assisted selection efficiency for high Pi/low phytate and low stachyose traits, 2) select and evaluate the yield potential and stability of low phytate/low stachyose soybean breeding lines, 3) study the planting date effects on soybean seed composition, 4) study the effects of delayed harvest on soybean seed composition, and 5) evaluate the potential relationship between soil properties and soybean leaf inorganic element concentration, and seed composition.

Implications and potential benefits to producers and the soybean industry

The implications of this research and potential benefits to soybean producers are: 1) develop the first low phytate/low stachyose soybean breeding lines/cultivar adapted to Arkansas, 2) understand how we can develop and deliver those breeding lines faster to the producers (increase selection efficiency), and 3) deliver specialty soybean cultivars that not only come with the desired traits, but also with supporting information on important management practices (planting date, time of harvest, potential response to specific soil nutrients) that may help producers meet marker specifications and consequently increase farm revenue.

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Table 1. Genetic sources of low phytate and/or low stachyose in soybean.

Genotype	Seed composition†					Enzyme	Mutation§	LG¶	Reference
	Suc	Gal	Raf	Sta	Phy				
PI 200508	High	High	Low	Low	Normal	Raffinose Synthase	3 bp deletion. Position 991-993 Triptophan deletion. *Rsm1* marker.	C2	(Dierking and Bilyeu, 2008; Skoneczka et al., 2009)
LR 33	High	--	Low	Low	Low	Mips1‡	1 bp mutation. G to T. 1888 bp from start codon.	B1	(Sebastian et al., 2000; Hitz et al., 2002)
Gm-lpa-TW-1	High	--	Low	Low	Low	Mips1	2 bp deletion. Frame shift.	B1	(Yuan et al., 2007; Yuan et al., 2009)
Gm-lpa-ZC-2	Normal	--	Normal	Normal	Low	Unknown	Unknown. Flanked by Satt168 and Satt416.	B2	(Yuan et al., 2007; Yuan et al., 2009)
V99-5089	High	--	Low	Low	Low	Mips1	1 bp mutation. G to C. Shift from Hist to Asp. Marker Satt453.	B1	(Saghai Maroof and Buss, 2011)
CX1834	Normal	--	Normal	Normal	Low	Multi-drug resistant protein	Non-sense mutation (A to T). Truncated protein.	L	(Walker et al., 2006; Gillman et al., 2009; Saghai Maroof et al., 2009)
CX1834	Normal	--	Normal	Normal	Low	Multi-drug resistant protein	Missense mutation. Amino acid substitution.	N	(Walker et al., 2006; Gillman et al., 2009; Saghai Maroof et al., 2009)

† -- = Not reported, - = Not detected, Suc = sucrose; Gal = galactinol; Raf = raffinose; Sta = stachyose; Phy = phytate.

‡ D-*myo*-inositol 3-phosphate synthase 1.

§ bp = base pairs; Hist = histidine; Asp = asparagine.

¶ linkage group.

II. Selection efficiency of low phytate and low stachyose genotypes from soybean breeding populations with different genetic backgrounds

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Abbreviations: Pi, inorganic phosphorus; *Lpa1*, low phytate gene 1; *Lpa2*, low phytate gene 2; *Mips*, D-*myo*-inositol 3-phosphate synthase 1 gene; mips, *myo*-inositol phosphate synthase mutant individual; WT, wild-type; mut, mutant; LG, linkage group; SSR, simple sequence repeat; SNP, single nucleotide polymorphism; MAS, marker-assisted selection; HPLC, high-performance liquid chromatography; KASPar, KBiosciences competitive allele specific PCR; PCR, polymerase chain reaction; Pop, population; bp, base pair; QTL, quantitative trait loci; FAM, 6-carboxy-fluorescine.

ABSTRACT

Rapid and efficient selection of genotypes in early generations of a breeding scheme is an important component of a crop breeding program. The development of soybean genotypes with improved seed quality attributes and reduced seed antinutritional components is necessary for producing animal feed with increased metabolizable energy and nutrient availability. In this study, a new method (KASPar system) for genotyping populations segregating for low phytate genes *Lpa1* and *Lpa2* was tested, and marker-assisted and phenotypic selection for the high inorganic phosphorus and low stachyose traits controlled by *Lpa* and *Mips* mutations were studied. Results showed that the KASPar system worked accurately, and can be used to effectively genotype breeding populations segregating for *Lpa* genes. Phenotypic selection for low phytate individuals containing *Lpa* mutations using the Pi assay was reliable. However, parental genotypes should always be included in the assay to construct thresholds before performing selections, as the effect of *Lpa* mutant alleles on Pi depends on the genetic background. On the other hand, phenotypic selection for mutants for *Mips* using Pi should be complemented with sugar data. Selection based solely on Pi may be inefficient, as this trait is not environmentally stable in *Mips* germplasm. Marker-assisted selection efficiency depended on the pedigree of the population. Molecular marker Satt561 captured all the high Pi individuals, but inheriting the mutant allele for the marker was not indicative of a high Pi phenotype, as three individuals of the seven carrying the mutant parent allele at Satt561 were not high Pi. Recombination can be a factor reducing simple sequence repeat marker-assisted selection efficiency. The KASPar system may be appropriate for laboratories that are well-established and preferably have an automated system to perform DNA extraction and handle samples. Methods that measure phytate, instead of Pi, may be a satisfactory alternative to molecular methods.

INTRODUCTION

About 80% of the soybeans produced in the Unites States are used, after being crushed for oil extraction, to produce soybean meal, a major component of animal feed. Phytate is the form in which most (70%) of the phosphorus (P) is stored in the soybean seed (Raboy, 2001). Phytate chelates essential minerals and makes them unavailable for absorption by animals consuming soybean meal (Raboy, 2009). Further, undigested phytate increases the concentration of P in manure derived from livestock. Land fertilization with this manure with high levels of P may result in eutrophication of surface and ground water due to excess P in farms run-off water (Ertl et al., 1998). Therefore, development of low phytate soybeans will increase the nutritional value of soybean meal (more minerals available) and will contribute to sustainable agriculture (reduced water pollution).

There are two genetic sources for the low phytate trait that have been widely used in public soybean breeding programs: CX1834 and V99-5089. CX1834 is a low phytate breeding line derived from the cross between 'Althow', a commercial cultivar, and M153, a low phytate line developed by chemical mutagenesis at Purdue University (Wilcox et al., 2000). The low phytate in CX1834 is controlled by two recessive quantitative trait loci (QTL), *Lpa1* and *Lpa2*. These two QTL were mapped by Walker et al. (2006) and confirmed in Virginia (Gao et al., 2008) and Tennessee (Scaboo et al., 2009) locations. *Lpa1* and *Lpa2* are located on LG N and L, and are linked to simple sequence repeat (SSR) markers Satt237 and Satt561, respectively (Walker et al., 2006; Scaboo et al., 2009). Sequence analysis has shown that *Lpa1* and *Lpa2* are two gene homologues that code for multi-drug resistance proteins that may be involved in cell guard functions (Gillman et al., 2009). *Lpa1* contains a single nucleotide nonsense mutation that results in a truncated protein, whereas *Lpa2* contains a missense mutation that results in an amino acid

substitution (Gillman et al., 2009). *Lpa* mutant alleles have been incorporated in adapted high-yield breeding lines (e.g. S04-0453-05 at Missouri, 04-05N32 at North Carolina) (Maupin et al., 2011b). V99-5089 is a low phytate line developed by Virginia Polytechnic Institute and State University (Gao et al., 2008; Saghai Maroof et al., 2009; Maupin et al., 2011a) that contains high sucrose (75-90 mg g^{-1}), low raffinose (5 mg g^{-1}), and low stachyose (5 mg g^{-1}) (Florez-Palacios, 2009). The phytate levels of V99-5089 are not as low as those of CX1834 (Gao et al., 2008). V99-5089 contains a single nucleotide mutation (Saghai Maroof and Buss, 2011) in the D-*myo*-inositol-3-phosphate synthase 1 gene (*Mips*), which is in the convergent step of sugar and phytate synthesis, and thus responsible for the concomitant modification of the phenotypic expression of these two traits (i.e. low phytate and low stachyose). The high sucrose might have been a result of the much reduced stachyose in this particular genotype. The *Mips* gene is located on LG B1, and is linked to SSR marker Satt453 (Maupin et al., 2011a; Saghai Maroof and Buss, 2011). Like the *Lpa* mutant alleles, the *Mips* mutant allele has been incorporated in adapted lines (e.g. V03-5906, V03-5901 at Virginia Polytechnic Institute and State University, R07-2000 at University of Arkansas).

Because seed phytate and seed inorganic phosphorus (Pi) are negatively correlated in low phytate lines derived from both low phytate germplasm sources (Hitz et al., 2002; Scaboo et al., 2009; Maupin et al., 2011b), a commonly used and relatively inexpensive method of selection for low phytate lines is the measurement of seed Pi. Maupin et al. (2011b) studied two sets of low phytate lines; one derived from CX1834 and a second from V99-5089, grown in 12 environments (combinations of years and locations). Maupin et al. (2011b) found a significant genotype by environment interaction for phytate concentration and Pi, and reported that Pi was not as stable across environments as compared to phytate concentration. However, changes in

line ranking were not very common, and the average Pi concentration of each low phytate lines was always significantly higher than that of normal phytate lines. Thus, the Pi assay is considered an adequate and reliable method for identifying low phytate genotypes segregating for *Lpa* and *Mips* genes. Further, the Pi assay has been used to select low phytate lines in other low phytate germplasm, *Gm-lpa*-TW-1and *Gm-lpa*-ZC-2 (Yuan et al., 2007), that carry similar mutations to the ones in CX1834 and V99-5089.

Molecular marker-assisted selection (MAS) consists of indirectly selecting breeding lines for specific traits using linked DNA markers, but without growing the lines and measuring the phenotype of interest (e.g. yield, phytate, sugar). Quantitative trait loci have been found to be population (genetic background) or environment specific for many quantitative traits of importance (Brummer et al., 1997; Fasoula et al., 2004), so having QTL which effects are large, common and consistent across environments and populations is the best alternative to exploit marker-assisted selection (Bernardo, 2008). Thus, validating previously found QTL in different genetic backgrounds and environments helps determine the width of the spectrum in which molecular markers can be used to perform efficient and accurate breeding selections. Research focused on the search for this type of QTL should constitute a parallel enterprise to the breeding program (Bernardo, 2008).

Marker-assisted selection efficiency for low phytate genotypes has been studied in populations derived from both CX1834 and V99-5089. Scaboo et al. (2009) studied two populations of recombinant inbred lines segregating for *Lpa1* and *Lpa2* and calculated marker-assisted selection efficiency for high Pi genotypes using Satt237 (linked to *Lpa1*) and Satt561 (linked to *Lpa2*). Scaboo et al. (2009) reported an overall (populations and marker combined) 50% marker selection efficiency, given by the percentage of individuals that inherited the

CX1834 allele at both marker loci and also exhibited the high Pi phenotype. Selection with these markers captured all high Pi individuals, but carrying the CX1834 allele at both marker loci did not necessarily result in a high Pi genotype (Scaboo et al., 2009). Unlike Scaboo et al. (2009), Gao et al. (2008) found a perfect correlation between phenotypic (phytate-based) and genotypic (marker-based) selection in a breeding populations segregating for the *Lpa* genes. Scaboo et al. and Gao et al. proposed that additional QTL for low phytate may be contributing to the phenotype in the populations they studied. Therefore, selection with only these two SSR markers may not always be sufficient to capture all the high Pi (Gao et al., 2008). Maupin et al (2011a) studied a population of 153 recombinant inbred lines derived from the cross V99-5089 by Essex, a conventional high yield line. Maupin et al (2011a) reported 87% marker-assisted selection efficiency when using SSR marker Satt453, as 10 of 76 lines scored as homozygous for the low phytate allele were identified as low Pi instead. Further, there were 10 lines that showed the *Mips* phenotype but were identified as homozygous for the wild-type allele at Satt453. The lack of perfect selection efficiency was attributed to recombination between the marker and the gene controlling the trait.

Saghai Marrof et al. (2011) discovered the *Mips* mutation (single nucleotide) using DNA sequencing and sequence alignment techniques. Rosso et al. (2011) introduced a new method called KBiosciences (Hoddesdon, UK) competitive allele specific PCR (KASPar) to genotype soybean lines segregating for *Mips*. The KASPar system uses PCR amplification of alleles and specific primers, and fluorescence techniques to differentiate genotypes at the nucleotide level. Rosso et al. (2011) reported a 100% efficiency of the technique to classify individuals in populations segregating for the *Mips* mutation. The availability of this perfect marker at the gene of interest facilitates the estimation of phenotypic selection efficiency. Because perfect makers

are within the coding region of the gene, recombination between marker and the gene is not a factor that may account for reduced efficiency. This is, selection with perfect markers for the target trait would not produce false positives. Therefore, the number of lines that have the alleles (determined by perfect markers) coding for the low phytate trait and are also classified as low phytate with the Pi assay, will be a measure of phenotypic selection efficiency. Any difference between these two could only (assuming no error in genotypic and phenotypic scoring) be attributed to variation in Pi caused by non-genetic effects. The KASpar system has not been used to genotype individuals in populations segregating for the low phytate genes *Lpa1* and *Lpa2*. Incorporating this technique for genotyping plant populations segregating for *Lpa1* and *Lpa2* should represent a valuable asset for soybean breeders selecting low phytate individuals from large numbers of progenies, as it would allow for standardizing genotyping to a common platform independent of the source of the low phytate mutation.

The hypotheses of this study were that phenotype and marker-assisted selection will differ in their efficiency at selecting high Pi/low stachyose genotypes and the efficiency of marker-assisted selection will depend on the genetic background in which the high Pi/low phytate mutations are expressed.

The objectives of this study were to 1) evaluate the KASPar system to identify high Pi individuals carrying the *Lpa1* and *Lpa2* mutations, 2) determine phenotypic selection efficiency for two breeding populations segregating for *Lpa1* and *Lpa2* and *Mips* mutations, respectively, and 3) determine SSR marker-assisted selection efficiency for low phytate genotypes in four breeding populations segregating for *Lpa1* and *Lpa2* mutations.

MATERIALS AND METHODS

Population development and field experiment

Six F_2 populations segregating for the low phytate trait were used in this study (Table 1). Crosses were made at the Arkansas Agricultural Research and Extension Center in Fayetteville, AR in the summer of 2009. The F_1 plants were grown in Fayetteville during the summer of 2010 and true hybrid plants (not selfed) were verified using morphological markers and then bulk harvested. Second generation (F_2) seed from each population was planted in eight 5-meter rows with 150 seeds per row. Populations 2 and 6 were planted in Fayetteville, AR on May 18th 2011, whereas the other populations were planted at the Vegetable Research Station in Kibler, AR on June 1st 2011. A 3-m row of the parental genotypes was planted next to the segregating populations. Row spacing was 100 cm and 90 cm in Fayetteville (planted on beds) and Kibler (planted on flat ground), respectively. Field plots were fully irrigated with overhead at Kibler and by furrow irrigation at Fayetteville, and managed during the growing season using standard cultural practices adopted for full-season soybean production in Arkansas (Tacker and Vories, 1998). Cultural practices included tillage with chisel plow and disc and fertilization based on soil test results and recommendation of University of Arkansas Cooperative Extension Service. Weed control was performed by applying pre-plant and post-emergence herbicides (e.g. glyphosate), at label rates. The soil at the Fayetteville location is mapped as Captina silt loam (fine-silty, siliceous, active, mesic Typic Fragiudult) (Soil Survey Staff, 2011) and described as very deep, moderately well-drained soil developed on a thin mantle of silty material. The previous crop at the Fayetteville field was corn (*Zea mays*). At Kibler, the soil is mapped as Roxana silt loam (coarse-silty, mixed, superactive, nonacid, thermic Typic Udifluvents) (Soil Survey Staff, 2011) and described as a well-drained, moderately permeable soil formed in stratified loamy alluvium (Garner and Cox, 1979). The previous crop at the Kibler field was soybean. Four weeks after planting, about 130 plants (except for population 1) were tagged and identified with a number on

the plastic tag. Most recently developed trifoliate leaf samples from each tagged plant were collected for DNA extraction. Segregation for flower and pubescence color was examined to ensure the population was derived from a true cross as intended. At maturity, tagged plants were threshed individually and the seed was stored for Pi and/or sugar analysis. Plants of parental genotypes were also sampled for DNA extraction and seed was harvested for Pi and sugar analysis.

Genotypic data

DNA extraction. Plant tissue was ground with liquid nitrogen (N) to a fine powder using a mortar and a pestle. DNA was extracted with CTAB extraction buffer and chloroform: isoamyl alcohol (24:1). Afterwards, DNA was precipitated and washed with 95% ethanol, and resuspended in TE buffer (Kisha et al., 1997). DNA concentration of each sample was calculated by measuring absorbance at 260 nm in a plate reader (Biotek, Winooski, VT). Then, DNA was diluted to a concentration of 7 ng/µl.

Single nucleotide polymorphism (SNP) genotyping with KASPar system. Individuals were genotyped for the *Lpa1* (Pop1, Pop2, Pop3, Pop4,Pop 5), *Lpa2* (Pop1), and *Mips* (Pop6) mutations using the KASPar system at Virginia Polytechnic Institute and State University, Blacksburg, VA by Luciano M. Jaureguy, following the procedures described by Rosso et al. (2011). Briefly, a DNA sequence of about 50 base pairs (bp) flanking the single-nucleotide mutations for *Lpa 1*, *Lpa2*, or *Mips*, was submitted to KBiosciences (Hoddesdon, UK) for primer design (Table 2). KASPar reaction mix consisted of 5 µl of DNA (7 ng/µl), 5 µl of 2x KASPar reaction mix and 0.14 µl of primers (two 5' fluor-labeled allele-specific primers, one for each allele at the mutation site, and one common reverse primer) (KBiosciences, UK). Thermocycling conditions consisted of hot start enzyme activation, 15 min at 94°C, followed by 10 cycles of

melting at 94°C for 20 s, and annealing from 65-57°C (dropping 0.8°C per cycle), followed by 15 cycles of 10 s at 94°C and 60 s at 57°C. Polymerase chain reactions and fluorescent endpoint reading were performed in a 7,300 Real-Time PCR System (Applied Biosystems, USA). After PCR were completed a post-read run was performed. Data were expressed in fluorescence signal versus filters, by converting it to pure dye components using the extracted pure dye standards. The contribution of each dye for each individual was plotted and allele manual calling was performed. Individuals that were between clusters were genotyped again until allele calling was unambiguous.

SSR genotyping. Genotyping was carried out by Dr. Luciana M. Rosso at Virginia Polytechnic Institute and State University, Blacksburg, VA. Individuals were genotyped with SSR markers Satt527 and Satt561 (Pop2, Pop3, Pop4,Pop5), previously reported to be the closest to the *Lpa2* gene (Walker et al., 2006; Gao et al., 2008; Gillman et al., 2009) and therefore the two most efficient SSR markers (Gillman et al., 2009) to select individuals carrying the mutant *Lpa2* allele. The samples were screened with the SSR VIC™ (green)-labeled marker Satt527 and SSR FAM (blue)-labeled marker Satt561. Polymerase chain reactions for the SSR assay were performed in a total volume of 12.5 μL in a Bio-Rad C1000 (Bio-Rad Laboratories, Hercules, CA) thermal cycler. The initial denaturing step, 5 min at 95°C, was followed by 40 cycles of 30 s at 94°C, 40 s at 47°C, 30 s at 72°C, and then by a final extension step for 7 min at 72°C. Genotypes were visualized by a 3130x*l* Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Scoring of the genotypes was done manually and based on allele size. Marker Satt527 amplicons were 198 bp (mutant allele) and 201 bp (WT allele), whereas Satt561 amplicons were 246 bp (mutant allele) and 240 bp (WT allele).

Phenotypic data

Sugar analysis. Seed from single plants was processed separately for sugar extraction, sugar fractionation and identification, and sugar quantification by high performance liquid chromatography (HPLC) as described in Hou et al. (2009). Briefly, 10 g of seed from each plant were ground in a coffee grinder (Krups®, Shelton, CT) and the powder was screened through a 450 μm sieve (VWR International, West Chester, PA). The fine powder (0.15 g) was placed in a 2.0 ml centrifuge tube. Filtered sterile deionized water (1.5 ml) was added; the tube was vortexed until the solution was homogenous, and shaken at 200 rpm for 20 min on a flat laboratory rotator (Thermo Scientific; Dubuque, IA). The sample was then centrifuged at 13500 *g* for 10 min, and 500 μl of the supernatant were transferred to a 1.5 ml tube containing 700 μl of acetonitrile. The solution was mixed by inversion and incubated at room temperature for 30 min to precipitate water soluble proteins. Afterwards, the sample was centrifuged at 13500 *g* for 10 min. The supernatant was filtered using 0.2 μm filter paper discs (Pall Lifesciences, East Hills, NY) mounted on 25 mm syringe filter holders (VWR International, West Chester, PA). Extracts were stored at 4°C until analysis by HPLC. An aliquot of 24 μl of sugar extract from each sample was diluted in 576 μl of sterile deionized water and loaded in the HPLC. The HPLC system used for this experiment was a Dionex DX500 (Dionex Corporation**,** Sunnyvale, CA) equipped with a GS50 pump, an LC50 chromatographic oven, and an ED40 electrochemical detector. The separation of the sugar was performed by a CarboPac PA 10 pellicular anion-exchange resin column (250 x 4 mm), coupled to a guard column (50 x 4 mm) preceded by an AminoTrap column (30 x 3 mm), contained in the chromatography oven, and maintained at 35°C. Sugars were eluted under isocratic (constant pressure) conditions with 90 Mm NaOH at a flow rate of 1 ml/min, and pressurized with ultrapure helium. The mobile phase 90 mM NaOH solution was prepared by diluting 50% (w/w) NaOH (VWR International, West Chester, PA) in deionized

water and degassed with gaseous N for 20 min. Sugar detection was achieved by pulsed-amperometric detection (Dionex Corporation, Sunnyvale, CA). Sugar standards for sucrose, raffinose and stachyose (Sigma-Aldrich, St. Louis, MO) were included at the beginning of each run to determine the retention time for each sugar and posterior identification of sugars in the samples. A sample of a solution of known concentration of sucrose (48 μg) and stachyose (30 μg), prepared by dissolving sugar in dedionized water and independently of the standards, was also included in each batch of samples as controls for sugar measure repeatability by the HPLC system. Peak area and sugar amount (μg) of each standard was used to build a standard curve for quantification. The standard amounts were: 10, 20, 40, 60, 80 μg of each sugar in 0.6 ml of water. Calculation of sugar concentration in samples was done by linear extrapolation using the formula μg sugar = area/curve slope. Sugar data were presented in milligrams of sugar per gram of seed and in on "as is" basis, which was determined by converting the HPLC reading (μg sugar) to milligrams of sugar per gram of seed (mg g^{-1}). The conversion factor was calculated according to the changes in concentration that sugar undergoes during the extraction process.

Pi analysis. Classification of individuals for the phytate phenotype was based on an inverse relationship between phytate and Pi that has been reported for lines derived from V99-5089, CX1834, and other low phytate germplasm sources (Yuan et al., 2007; Maupin et al., 2011a). A sample of 0.1 g of ground and sieved soybean seed powder was weighed in a 1.5 ml centrifuge tube. One ml of extraction buffer (deionized water, 12.5% trichloroacetic acid, 1 M $MgCl_2$) was added, and the solution was vortexed until it became homogeneous. The solution was incubated overnight (≈16 h) at 4ºC. Then, the sample was vortexed, incubated at room temperature for 25 min, and centrifuged at 1400 *g* for 4 min. Samples were stored at 4ºC until Pi analysis. A 10 μl sample of each extract was placed in a well of a flat bottom 96-well plate (Becton Dickinson,

Franklin Lakes, NJ), with each well containing 90 µl of deionized water. Each plate also included Pi standards (155 µg, 465 µg, 930 µg, 1395 µg, 1860 µg, 2325 µg, 2635 µg) which consisted of varying amounts of K_2PO_4 proportionally diluted in deionized water. Standards and samples were allowed to react with 100 µl of Chen's reagent (6 N sulphuric acid, 2.5% amomium molybdate, 10% abscorbic acid, deionized water) for 1 h, and absorbance at 882 nm was measured in a plate reader (Biotek, Winooski, VT). Samples were run in batches of 96 samples, and all the samples were run with the same batch of reagents. A sample of CX1834-1-6 (high Pi/low phytate) and Osage (low Pi/normal phytate) were also included in each batch of samples as controls for the extraction process. Data were presented as micrograms of P per gram of seed (µg g^{-1}) and on an "as is" basis.

Statistical analysis

Observed number of individuals for each genotypic class was compared to the expected number of individuals for a 1 (Pop6) and 2 (Pop1) gene models using goodness-of-fit Chi-square tests. Phenotypic selection efficiency for Pop1 was calculated as the number of double mutants (*Lpa1* and *Lpa2*) having high Pi divided by the total number individuals with high Pi and multiplied by 100. Phenotypic selection efficiency for Pop6 was calculated as the number of individuals carrying the *Mips* mutation and having high Pi/low stachyose divided by the total number of invdiduals having high Pi/low stachyose and multiplied by 100. The gene models represented by the marker data and phenotypic selection efficiency were tested only in populations 1 and 6 because these populations were genotyped with SNP markers at the genes of interests and were, therefore, free of false positives, which in other circumstances may be caused by factors such as recombination and environmental effects.

Marker-assisted selection efficiency for Pop2, Pop3, Pop4, and Pop5 was calculated as the number of individuals having both mutant *Lpa* alleles [based on SNP (*Lpa1*) and SSR markers (*Lpa2*)] and high Pi, divided by the total number of individuals selected with the SSR markers and multiplied by 100.

RESULTS AND DISCUSSION

KASPar system performance

The KASPar system was able to separate individuals in three distinct clusters and facilitate quick and easy genotype scoring (Fig. 1). Marker alleles segregated the wild-type vs. mutant alleles as expected. Chi-square tests using genotypic classes from the SNP data confirmed the two-gene (*Lpa1* and *Lpa2*) and single-gene (*Mips*) segregations models in population 1 and population 6, respectively (Table 3). The distribution of individuals in Pop 6 also fitted a phenotypic ratio of 3 (normal stachyose) to 1 (high Pi/low stachyose). These results indicated that the KASPar system can be used to accurately genotype and classify individuals of breeding populations segregating for *Lpa* genes into genotypic classes, and consequently used to select double *Lpa* mutants. Our results also confirm the capabilities of the KASPar system with populations segregating for the *Mips* gene previously reported by Rosso et al. (2011).

Phenotypic selection

Phenotypic selection for high Pi was effective, as the Pi assay correctly identified the only *Lpa* double mutant in the population (Table 3). The Pi concentration of this individual (3218 µg g^{-1}) was more than 50% higher than the second highest individual (2074 µg g^{-1}) in the population, and more than four times higher than the population mean (668 µg g^{-1}). Further, that individual was the only one that was in the same range of Pi value as the mutant parent Md06-5415 (3052 µg g^{-1}). Thus, the Pi assay is confirmed as a reliable method to identify *Lpa* double

mutant individuals. However, the chances of correctly selecting a true double mutant by using the Pi assay will be maximized if only individuals that significantly stand out from the population and that exhibit Pi concentration similar to the mutant parent are selected. If we had not included the mutant parent as reference, we could have ended up selecting individuals that, although having significantly higher Pi than the population mean, were not genetically double *Lpa* mutants.

Phenotypic selection for individuals carrying the mutant allele at *Mips* (mips) using Pi was not as effective in the population studied as anticipated. The average Pi concentration of the mips was 725 µg g^{-1} (Table 3). The highest individual Pi concentration was 1391 µg g^{-1}, which was lower than the parent (1654 µg g^{-1}) carrying the *Mips* allele. If phenotypic selection based on Pi concentration had been performed in this population, only a few lines would have been selected (data not shown) for high Pi concentration. These findings are not completely in agreement with other studies. Rosso et al. (2011) found that all the high Pi individuals also carried the *Mips* allele in a population derived from the cross V01-1693 x V03-5901. Interestingly, if the individuals of population 6 in this study are sorted by Pi concentration, all the individuals (except 2) carrying the mutant alleles appear in the top tier. A possible reason for the lower Pi in the mips than the high Pi parent is that environmental effects may have proportionally affected the individuals of this population resulting in lower Pi concentration without changing individual rankings. Maupin et al. (2011a) reported that the mean Pi concentration of a group of 69 mips was 1595 µg g^{-1} in 2008 and 2391 µg g^{-1} in 2009, which represents a 796 µg g^{-1} year to year difference. Further, Maupin et al. (2011b) reported below average Pi stability for three mips across 12 environments (location-year combinations). The Pi of these lines across the environments ranged from about 600 to 2000 µg g^{-1} (based on Fig. 1 of Maupin et al. 2011b).

Therefore, in a practical screening program, relative rankings of individuals under question and their comparison with reference checks are critical in phenotypic classifications. Therefore, although both mutations contribute to low phytate and high Pi, using the Pi assay to screen for progeny carrying mutant alleles might not be the best approach, especially if the source of the mutations (*Lpa* or *Mips*) is unknown. An adequate alternative would be to grown CX1834 and V99-5089 along with the segregating populations, and use the two lines to set a threshold and determine if a high Pi individual contains a *Mips* mutation or it is a double *Lpa* mutant. This would be of significant importance if molecular and sugar data are not available.

Stachyose distribution showed a bimodal distribution with two very distinct groups of individuals (Fig. 2) and three distinct genotypic classes (Table 3), which confirms the single recessive gene inheritance previously proposed for the *Mips* gene (Huhn, 2003; Florez-Palacios, 2009). Selection of mips based on stachyose distribution was 100% effective, as all and only the individuals in the low stachyose group contained the *Mips* mutation, as confirmed by the SNP marker analysis.

Marker-assisted selection

Population 4 showed the largest proportion of high Pi individuals (Table 4). Differences in germination of F_2 plants with and without the mutant alleles may have caused differences between the observed and expected number of high Pi individuals within and among populations. The low phytate trait is known to be accompanied by reduced germination and field emergence (Meis et al., 2003; Hulke et al., 2004; Oltmans et al., 2005), and it has been proposed to be responsible for skewed genotypic ratios in breeding populations segregating for the *Lpa* genes (Maupin, 2010; Maupin and Rainey, 2011). The wild-type parent of population 4 may contain alleles that counteract the effects of the low phytate mutant allele on germination and emergence

and thus resulting in the higher number of double mutants in this population. Therefore, the potential problem of low germination and field emergence with the low phytate trait can be overcome by using different genetic backgrounds, providing opportunity for breeders to incorporate the target trait of interest and eliminate any linked deleterious traits. Breeding lines such as 04-05N32 contain the *Lpa* mutations but exhibited acceptable germination (Maupin and Rainey, 2011), and are currently used for breeding low phytate soybeans.

Marker-assisted selection for high Pi individuals with SNP marker for *Lpa1* and SSR markers for *Lpa2* was 100% effective in the two populations that had TN09-239 as *Lpa1* and *Lpa2* donor parent, whereas it was 50% and 60% effective in the two populations derived from Md06-4615 as a low phytate parent (Table 4). Markers Satt527 and Satt561 were equally effective for identifying high Pi individuals in the TN09-239 population, but one of the high Pi individuals in the Md06-4615 populations inherited the WT allele at the Satt527 locus, which could likely be a result from crossover between the *Lpa* gene and the SSR marker leading to genetic recombination. It appears that Satt561 has a wider application in terms of selection in diverse germplasm and it is confirmed as the best SSR marker to select mutant individuals for the *Lpa2* gene.

The three selection systems [KASPar, Pi, sugar data for mips)] presented in this study have important implications in conventional (phenotype-assisted) and molecular breeding programs, particularly from the standpoints of efficiency, cost, and labor. The efficiency of the KASPar system for selecting *Lpa* and *Mips* mutant was, as shown by Rosso et al. (2011) and the present study, highly superior to using Pi or sugar data. Although the genotyping is relatively quick and the user will only need basic knowledge on molecular techniques, the leaf sampling, and DNA extraction and dilution may hinder the KASPar from being the most time efficient method. Thus,

the KASPar system may be appropriate for laboratories that are well established and preferably have an automated system to perform DNA extraction and handle samples. Using Pi to select *Lpa* and *Mips* mutant may be a satisfactory alternative to the other two methods, but only if the appropriate controls are added to the assay. Considering the instability of Pi in *Mips* germplasm across environments, the addition of CX1834, V99-5089, and a normal Pi control in the field experiment is strongly recommended in order to determine selection thresholds. The Pi assay is relatively cheap, requires few seeds, and once samples are ground, a trained individual can run 200 samples in a day. Using sugar data to select mips should be, as show in the present study, highly efficient. A disadvantage of this method may be the labor involved in extracting and quantifying sugars. Typically, at the University of Arkansas soybean breeding laboratory, up to 72 samples a day can be extracted, but it would take 1.5 d to run them in the HPLC system. On the other hand, if the *Mips* mutation is the main genetic source of low phytate/low stachyose used in the breeding program, using stachyose data is probably the best way to select mutant individuals, and assuming that the cost and labor of sugar analysis outweighs those of doing SNP genotyping.

CONCLUSIONS

Selection for low phytate lines carrying *Lpa1* and *Lpa2* mutant alleles can be performed using the Pi assay, but only if a well-defined cutoff point is pre-established and parental lines are used as a selection reference. Breeders screening progenies with different pedigrees and therefore not having a trait distribution may have to identify mips using the original mutants (CX1834, V99-5089) as reference. The different nature of the *Lpa1*, *Lpa2* and *Mips* mutations and their effect on seed composition may determine which selection method is more convenient for breeding for the low phytate or low stachyose trait. Phenotypic selection for mips using only

the Pi assay may not be reliable due to the instability of Pi in V99-5089-derived germplasm. Contrarily, selection of mips using stachyose concentration should be highly effective. Especially considering that a population segregating for the *Mips* gene should, unless significant additive effects derived from the non-mips parents exist, exhibit a bimodal distribution for stachyose concentration. The availability of SNP markers for *Lpa* and *Mips* genes represent an excellent alternative for screening large number of progenies with high efficiency. The fact that most of the public breeding programs use *Lpa* and *Mips* as their sole genetic source of low phytate and low stachyose makes these markers even more valuable. Perhaps, and in order to cope with the instability of Pi, breeders not being able to afford marker-assisted selection, should instead of measuring Pi, opt for recently developed methods that measure phytate (Gao et al., 2007; Burleson et al., 2012)

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Table 1. Description of the soybean F_2 populations used in this study.

Population designation	Pedigree	Source of low phytate and/or low stachyose	Markers screened		No. individuals screened	Location
			SNPs†	SSR‡		
P1	R07-743 x Md 06-5415	Md 06-5415	*Lpa1*, *Lpa2*	-	128	Kibler
P2	R06-4433 x Md 06-5415	Md 06-5415	*Lpa1*	Satt527/Satt561	70	Fayetteville
P3	R07-9019 x Md 06-5415	Md 06-5415	*Lpa1*	Satt527/Satt561	130	Kibler
P4	TN09-239 x R05-3239	TN09-239	*Lpa1*	Satt527/Satt561	130	Kibler
P5	TN09-239 x Osage	TN09-239	*Lpa1*	Satt527/Satt561	130	Kibler
P6	R07-7645 x R07-2002	R07-2002	*Mips*	-	130	Fayetteville

† *Lpa1*, *Lpa2* = Low phytate genes 1 and 2, respectively. *Mips* = D-*myo*-inositol 3-phosphate synthase 1 gene.

‡ - = no SSR marker screened for that population. Satt527/Satt561 are tightly linked to *Lpa2* (Walker et al., 2006; Gao et al., 2008) and were used to genotype parents and progeny.

Table 2. DNA sequence submitted to Kbiosciences for primer design for SNP marker screening for the low phytate (*Lpa1*, *Lpa2*) or low phytate/low stachyose (*Mips*) mutants in soybean.

SNP	Sequence†		
Lpa1	TTTCAGATCAAAAAGCAATTAAAGAGAAGAAGAAGAAAGCAAAACGATCG**[A/T]** GA AAGAAACAGCTTGTTCAGGAAGAGGAGAGGATTAGAGGTAGAGTCAGC	A = WT‡	T = LP mutant
Lpa2	GTGGATACTCAGCTTTTGCAAACTTGAAAATAAAATTATATCTATTGAGA**[G/A]** AATTTATCAGTACAGCCAAATTCCTAGTGAAGCACCCACAGTTATTGAAG	G = wt	A = LP mutant
Mips	GATGATATGGTCAACAGCAATGCCATCCTCTATGAGCCTGGTGAACATCCAGAC**[C/G]** AT GTTGTTGTTATTAAGGTAAATTTTGTTTCACCCATTTTTCTGTTTCTTTCTCTT	C = WT	G = LP mutant

† Single strand sequence. After the polymorphic nucleotide site (e.g. [A/T]), sequence continues in the next line on the right side.

‡ WT = wild-type; normal levels of phytate/low Pi; LP = low phytate/high Pi.

Table 3. Chi-square (χ^2) test for goodness-of-fit of observed and expected number of individuals for genotypic classes of two F_2 populations segregating for *Lpa* (Pop 1, 2 gene model) and *Mips* (Pop 6, 1 gene model) genes, respectively.

Population 1

Markers		**No of individuals**		
Lpa1	***Lpa2***	**Expected**	**Observed**	**Pi ¶ (µg g^{-1})**
WT†	WT	7	13	273
WT	Het	15	12	283
WT	mut	7	8	354
Het	WT	15	13	313
Het	Het	30	37	599
Het	mut	15	14	932
mut	WT	7	3	372
mut	Het	15	17	1495
mut	mut	7	1	3218
Total No. if ind			118	
Mean				668
SD‡				491
Max				3218
Min				231
Mut parent				3053
WT parent				252

P# χ^2obs (15.5)>χ^2crit (15.51) = 0.05

Table 3. Continued.

	Population 6					
	Marker	**No of individuals**		**Phenotype¶**		
	Mips	**Expected**	**Observed**	**Pi (µg g^{-1})**	**Suc (mg g^{-1})**	**Raf (mg g^{-1})**	**Sta (mg g^{-1})**
	WT	32	32	215	56	9	36
	Het	64	69	307	63	8	30
	mut	32	27	725	81	2	4
Total No. of ind.			128				
Mean				372	65	7	26
SD ‡				230	12	3	12
Max				1391	105	15	43
Min				143	46	1	0
Mut parent				1654§	99	1	3
WT parent				221	50	4	30
	P# χ^2obs (1.2) >χ^2crit (5.9) = 0.55						

† WT = wild-type; Het = heterozygous; mut = mutant. .

‡ SD = standard deviation; Max = maximum value; Min = minimum value.

§ Due to unavalability of seed of R07-2002, a sister line R07-2000 was used as reference.

¶ Pi = inorganic phosphorus concentration; Suc = sucrose concentration; Raf = raffinose concentration; Sta = stachyose concentration.

Probability of the χ^2obs was equal or larger than the χ^2crit with 8 (Table A) and 3 (Table B) degrees of freedom. χ^2Obs = Chi-square observed; χ^2Crit = Chi-square critic (from Chi-square table).

Table 4. Genotype and inorganic phosphorus (Pi) levels of individuals that were scored as high Pi/low phytate using molecular markers in four F_2 breeding populations segregating for the low phytate trait.

	Pop2				Pop3			
	***Lpa1*†**	***Lpa2* (Satt527)**	***Lpa2* (Satt561)**	**Pi (µg g^{-1})**	***Lpa1***	***Lpa2* (Satt527)**	***Lpa2* (Satt561)**	**Pi (µg g^{-1})**
	mut§	**mut**	**mut**	**3062**	**mut**§	**mut**	**mut**	**3434**
	mut	mut	mut	2644	**mut**	**mut**	**mut**	**3431**
					mut	**WT**	**mut**	**3357**
					mut	mut	mut	2546
					mut	mut	mut	2543
Mut parent‡				2926				3014
WT parent				235				245
Marker selection efficiency			50%				60%	

Table 4. Continued.

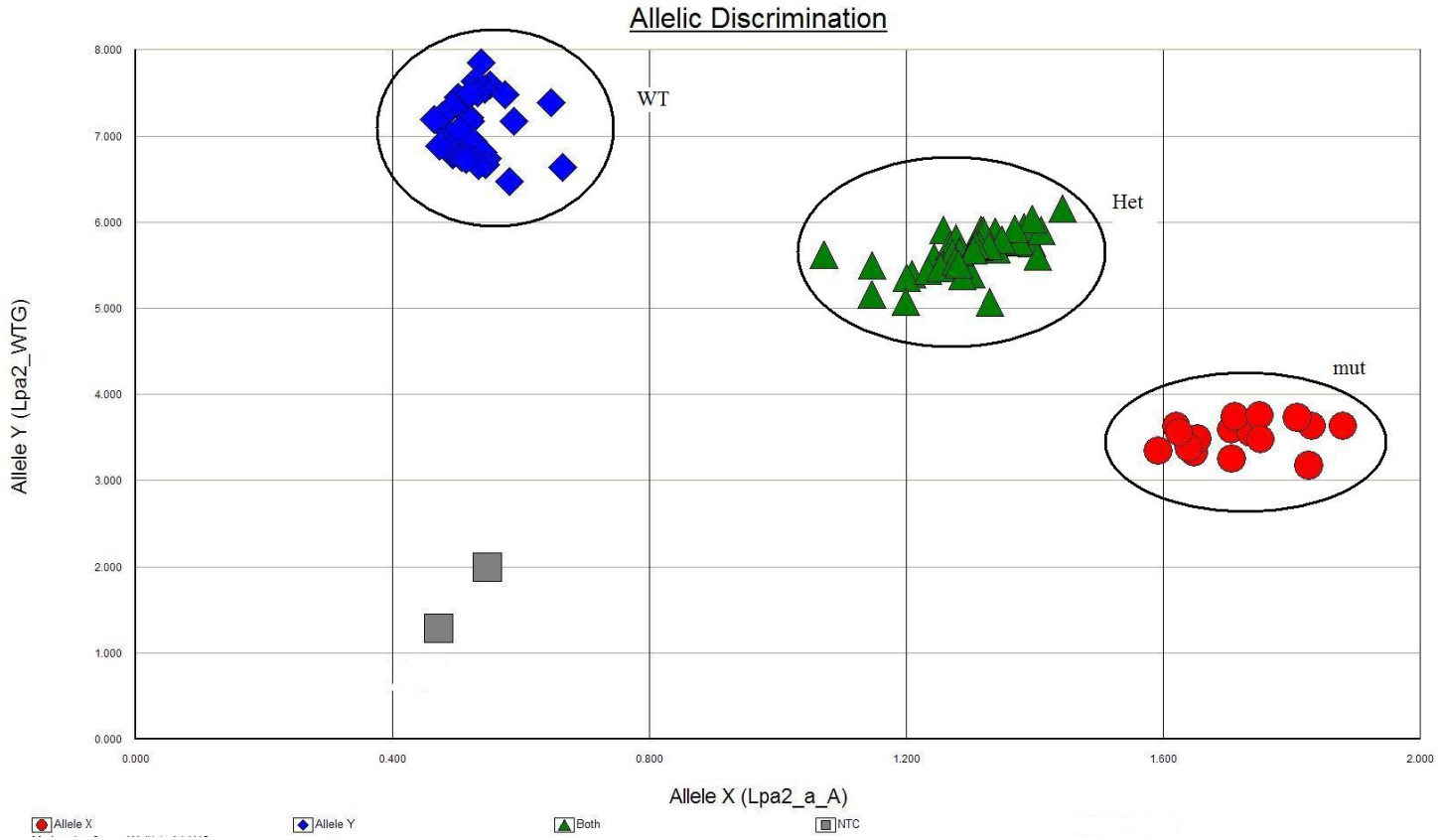
	Pop4				Pop5			
	***Lpa1*†**	***Lpa2* (Satt527)**	***Lpa2* (Satt561)**	**Pi (µg g^{-1})**	***Lpa1***	***Lpa2* (Satt527)**	***Lpa2* (Satt561)**	**Pi (µg g^{-1})**
	mut§	**mut**	**mut**	**2940**	**mut§**	**mut**	**mut**	**3554**
	mut	**mut**	**mut**	**2851**	**mut**	**mut**	**mut**	**3305**
	mut	**mut**	**mut**	**2835**	**mut**	**mut**	**mut**	**2421**
	mut	**mut**	**mut**	**2752**				
	mut	**mut**	**mut**	**2587**				
	mut	**mut**	**mut**	**2562**				
Mut				2023				2055
WT parent				242				197
Marker selection efficiency				100%				100%

† *Lpa1* was genotyped with SNP marker; *Lpa2* was genotyped with linked SSR markers Satt527 and Satt561. WT = homozygous for wild-type allele; Het = heterozygous; mut = homozygous for the mutant allele.

‡ Mut parent is homozygous for the mut allele at the three loci. WT parent is homozygous for the WT allele at the three loci.

§ Individuals in bold font were scored as high Pi/low phytate by the Pi assay.

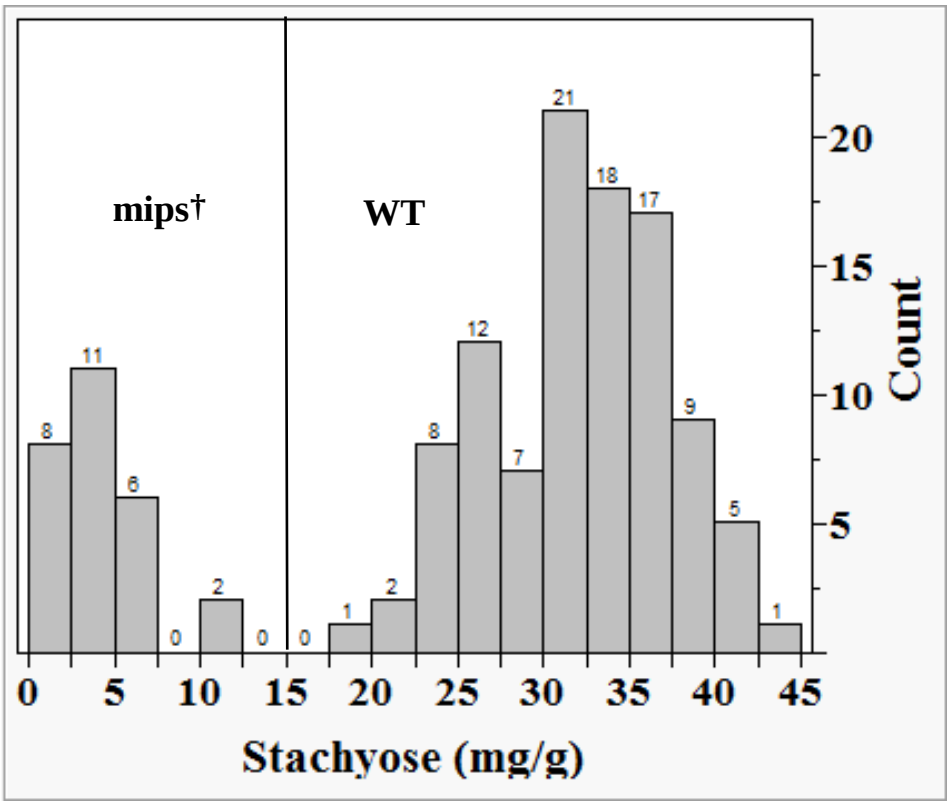
Figue 1. Genotypic classification by the KASPar SNP assay for *Lpa2* of a subset of 96 individuals of population 1 segregating for the low phytate trait. Allele specific primer 1 for wild tye allele is represented by FAM (blue), allele-specific primer 2 for mutant allele is represented by VIC™ (red), and heterozygous lines are represented in green.



† WT = wild-type allele; Het = heterozygous alleles; mut = mutant allele.

The two gray squares are the two no template controls, in which the same precedures were followed but no DNA was added to the reaction.

Figue 2. Distribution of stachyose concentration for the 128 individuals from population 6.



† mips (left panel of the distribution) included all and only individuals carrying the *Mips* mutation. WT (right panel of the distribution) included wild-type individuals and heterozyous individuals carrying wild-type and both alleles, respectively.

Numbers on bars indicate the number of individuals whose stachyose concentration fell within the range delimited by the bar.

The distribution of individuals fitted a genotypic ratio of 1 (homozygous dominant) to 2 (heterozygous) to 1 homozygous recessive (mips), and also a phenotypic ratio of 3 (normal stachyose) to 1 (low stachyose).

III. Selection and evaluation of high inorganic phosphorus-low stachyose soybean breeding lines in Arkansas

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Abbreviations: HPLC, high Performance liquid chromatography; *Mips*, D-*myo*-inositol 3-phosphate synthase 1 gene; P, phosphorus; Suc, sucrose; Sta, stachyose; Ole, oleic acid; Lin, linolenic acid; Sats, saturated fatty acids; Pi, inorganic phosphorus; CK, check cultivar; dfn, degrees of freedom of numerator; dfd, degrees of freedom of denominator, LSD, least significant difference

ABSTRACT

Soybean seed stachyose and phytic acid are not digested by monogastric animals, and thus represent an obstacle for an efficient utilization of soybean meal in animal feed. Land fertilization with animal manure derived from animal feed containing soybean can result in eutrophication of surface and ground water. The objectives of this research were to select breeding lines with high inorganic phosphorus/low phytate, high sucrose, and low stachyose from an $F_{2:6}$ breeding population derived from the cross S02-529 x V99-5089, and evaluate their yield potential and stability. Sucrose appeared to follow a normal distribution and stachyose and Pi exhibited a bimodal distribution, however, only stachyose and Pi satisfactorily fitted to the 3:1 ratio as expected for one recessive gene model previously reported for the low stachyose/high Pi trait in V99-5089. None of the selected lines performed agronomically better than the control cultivars. The highest yielding line, R08-6023 (2894 kg ha^{-1}), showed relatively low stability across environments, but it would be an adequate choice for highly productive environments. R08-6009 ranked fourth in overall yield and less yield fluctuations than other lines studied. One of the selected lines had moderately high oleic acid concentration (>45 mg g^{-1}), and another three lines had low linolenic acid concentration (<34 mg g^{-1}), which are novel and unique combinations with the high Pi/low stachyose trait. Although relatively low in yield potential as compared to the commercial checks, the breeding lines presented here could be incorporated in breeding programs as a source of those quality trait combinations. These multiple trait stacks would be of particular value for commercialization when they are incorporated into high-yielding backgrounds, which may give farmers market-competitive cultivars.

INTRODUCTION

Stachyose and other seed polysaccharides are involved in seed desiccation resistance (Black et al., 1996; Obendorf, 1997), transport of sugar in the phloem (McCaskill and Turgeon, 2007) and function as storage reserves and cryoprotectants in frost-hardy plant organs in some plants (Pennycooke et al., 2003). Phytic acid is the form in which most (70%) of the phosphorus (P) is stored in the soybean seed (Walker et al., 2006). Stachyose and phytic acid are considered antinutritional components of the soybean seed as they are not digested by monogastric animals, and thus represent an obstacle for a efficient utilization of soybean meal in animal feed. Stachyose is not broken down to simple sugars, so its nutritional value (metabolizable energy) is limited. Similarly, undigested phytate in the animal system increases the concentration of phosphorus in manure, which, if used in soil-applied fertilization, may lead to eutrophication of surface and ground water. Thus, screening for genotypes with improved seed composition is crucial for developing environmentally friendly soybeans with increased quality and nutritional value.

Two basic principles must be taken into account for developing quality trait cultivars. First and before embarking in a long and costly testing program, breeding lines should be tested for variation and presence of these traits. This can be done by conducting a phenotypic or molecular screening of preliminary lines or individuals plants early in the breeding process. Second, yield potential and yield stability should be evaluated before making selection or release decisions. Yield stability is important for developing cultivars adapted to different climatic and edaphic conditions (Beaver and Johnson, 1981) and, thus, breeders need to design breeding strategies for targeting cultivars for contrasting production environments. For example, cultivars that have an average or slightly below average performance in a wide variety of environments may be chosen

over cultivars that perform well in only a few environments (Schutz and Bernard, 1967; Beaver and Johnson, 1981). Seed quality traits, particularly those that have quantitative inheritance, and therefore may be more affected by the environment than qualitative traits, are also desired to exhibit stability to ensure profit if premiums are paid for improved seed composition.

A high inorganic phosphorus (Pi)/low phytate, high sucrose, low stachyose breeding line V99-5089 was developed by Virginia Polytechnic Institute and State University (Saghai Maroof and Buss, 2011). This line has a single nucleotide recessive mutation in the D-*myo*-inositol 3-phosphate synthase 1 gene (*Mips*), which yields a mutant enzyme that is unable to convert glucose 6-P to *myo*-inositol 1-P, a key substrate in the synthesis of phytate and sugar and thus the cause of the concomitant changes in those seed components (i.e. decreased phytate and stachyose accompanied with elevated sucrose). Such changed seed quality attributes have generated tremendous interest in the basic research and applied breeding communities. V99-0589 has been crossed to other lines in order to incorporate its unique traits into high-yield backgrounds (Maupin et al., 2011b), and combine them with other quality traits.

Most of the studies on yield potential of low phytate lines have been conducted using lines derived from CX1834, a low phytate mutant that carries two non-allelic recessive mutations that are different than the *Mips* mutation in V99-5089. Hulke et al. (2004) evaluated the yield of low phytate and reduced palmitic acid breeding lines in three locations in Iowa and reported no significant differences in yield compared to normal phytate lines. Low phytate lines in that study averaged 1634 kg ha^{-1} and the normal phytate lines averaged 2091 kg ha^{-1}. Oltmans et al. (2005) observed similar results in a study of low phytate lines selected from three populations and grown at the same locations and in the same year as Hulke et al. (2004). Low phytate lines exhibited an average yield of 1616 kg ha^{-1} and normal phytate lines 1993 kg ha^{-1}. Maupin and

Rainey (2011) evaluated six low phytate lines at six environments and reported that the average yield in CX1834-derived lines ranged from 3130 to 3457 kg ha^{-1}, and from 3046 to 3250 kg ha^{-1} in V99-5089-derived lines. Check cultivars 5601T and Essex had yields of 3970 and 3495 kg ha^{-1}, respectively. These studies showed that, although differences were not statistically significant, low phytate lines tended to yield less than normal phytate lines with competitive yield potential. In most, if not all, cases, low phytate lines yielded significantly less than the commercial check cultivars.

Although the yield potential of V99-5089-derived mutant lines has been recently studied (Maupin and Rainey, 2011), data on their yield performance in other genetic backgrounds and soybean growing environments are lacking. Considering that V99-5089 has a unique combination of quality traits and that V99-5089 has already been used in several breeding programs, data on yield stability and seed composition of high Pi, high sucrose, low stachyose lines derived from V99-5089 should be valuable for the development of widely adapted quality trait cultivars. In addition, it would be interesting and valuable to assess how well these traits combine with other seed quality attributes such as protein and fatty acids. Such information will be helpful to breeders in attempt to develop strategies for coping with multiple trait selections.

The objectives of this study were a) to identify high Pi/high sucrose/low stachyose lines from a population of 127 $F_{2:6}$ lines derived from the cross between a high-oleic acid line (S02-529) and V99-5089, and b) to determine yield, yield stability, and seed composition of the selected lines in five Arkansas environments.

MATERIALS AND METHODS

Field experiment

A cross between S02-529, a normal sugar and phytate, high-oleic acid breeding line and V99-5089 a high Pi/low phytate, high sucrose, low stachyose breeding line was made at the Arkansas Agricultural Research and Extension Center in Fayetteville, AR in 2004. Plat of generations F_1 and F_2 were grown at Fayetteville, AR in 2005 and 2006, respectively. Single F_2 plants were harvested in 2006, and subsequently, the F_2-derived lines were advanced to F_5 in Fayetteville, AR and in a winter nursery in Costa Rica by bulking the seed from each line in each generation. In the summer of 2009, 127 $F_{2:6}$ lines were grown in a randomized complete block design with two blocks containing 3-meter row plots at Fayetteville, AR and at the Northeast Research and Extension Center in Keiser, AR. The soil at the Fayetteville location is mapped as Captina silt loam (fine-silty, siliceous, active, mesic Typic Fragiudult) (Soil Survey Staff, 2011) and described as very deep, moderately well-drained soil developed on a thin mantle of silty material. The soil at the Keiser location is mapped as Sharkey-Steele complex (Sharkey: Very-fine, smectitic, thermic Chromic Epiaquerts; Steele: Sandy over clayey, mixed, superactive, nonacid, thermic Aquic Udifluvents) (Soil Survey Staff, 2011). Parental lines, S02-529 and V99-5089, were also included in the experiment as control and reference for the traits studied. Cultural practices in these and the following experiments included tillage with chisel plow and disc, and fertilization based on soil test results and recommendation of University of Arkansas Cooperative Extension Service. Weed control was performed by applying pre-plant and post-emergence herbicides (e.g. glyphosate), at label rates.

The entire row was of each line was harvested with a plot combine and seed was processed for phenotypic data. Sixteen lines were selected based on Pi concentration, stachyose concentration, and seed availability, and were advanced to yield trials grown at two and three Arkansas locations in 2010 and 2011, respectively. Locations in 2010 were the Rice Research

and Extension Center in Stuttgart, AR, and Keiser, AR. Locations in 2011 were Stuttgart, AR, the Pine Tree Research Experiment Station in Colt, AR, and the Southeast Research and Extension Center in Rohwer, AR. The soil at the 2011 locations (see above for Keiser soil type) are mapped as: Dewitt silt loam (Fine, smectitic, thermic Typic Albaqualfs) for Stuttgart, Calhoun silt loam (Fine-silty, mixed, active, thermic Typic Glossaqualfs) for Pine Tree, and Desha silt loam (Very-fine, smectitic, thermic Vertic Hapludolls) for Rohwer, respectively. In 2010, lines were grown in 5-m 2-row plots and arranged in a randomized complete block design with two blocks. At maturity, seed was harvested with a plot combine. Yield was measured in all the plots. Because it was only intended to assess the presence of the seed composition profile for which lines had been selected, sugar, Pi, protein, oil, and fatty acids were measured only in one plot per location. In 2011, lines were grown in 5-meter 4-row plots (except Rohwer; 5-row plots) and arranged in a randomized complete block design with three blocks. At maturity, the same procedures as in 2010 were followed. A seed sample from one block from Pine Tree and a sample from one block from Stuttgart were used for measuring protein, oil, seed size, and seed quality. Sugar and Pi were measured only in samples from Pine Tree.

Seed composition analysis

Protein and oil analysis. Samples containing 20-25 g of seed were sent to the USDA research facility at Peoria, IL or to the University of Missouri Delta Center at Portageville, MO for protein, oil and moisture analysis in a FOSS® (Eden Prairie, MN) near infrared transmittance instrument. Light of specific wave length interacts with the sample's organic compounds (e.g. protein and oil), which then transmit different light intensity depending on their concentration in

the sample. Protein and oil concentration were presented in milligrams per gram of seed (mg g^{-1}) and in dry-weight basis.

Fatty acid analysis. Samples containing five seeds were sent the DNA facility at Iowa State University in Ames, IA. Fatty acid concentrations [oleic acid, linolenic acid, and saturated fatty acids (sats)] were determined by gas chromatography according to the methods developed by Hammond (1991)**.** The fatty acid concentration was presented in milligrams per total grams of oil (mg g^{-1}) on an "as is" basis.

Sugar analysis. Each seed sample was processed separately for sugar extraction, sugar fractionation and identification, and sugar quantification by high-performance liquid chromatography (HPLC). For a detailed description on the HPLC methods see Jaureguy et al. (2012). Sugar data were presented in milligrams of sugar per gram of seed (mg g^{-1}) and on an "as is" basis.

Pi analysis. The rationale for this test is dependent on the inverse relationship between phytate and Pi which has been reported for lines derived from V99-5089 and other low phytate germplasm sources (Maupin et al., 2011a). Low phytate breeding lines generally exhibit high Pi (> 1000 µg g^{-1}) and normal phytate breeding lines low Pi. A sample of 0.1 g of soybean seed fine powder was weighed in a 2.0 ml centrifuge tube. One ml of extraction buffer (deionized water, 12.5% trichloroacetic acid, 1 M $MgCl_2$) was added, and the solution was vortexed until homogeneous. The solution was incubated overnight (≈16 h) at 4ºC. Then, the sample was vortexed, incubated at room temperature for 30 min, and centrifuged at 1400 *g* for 4 min. Samples were stored at 4ºC until Pi analysis. A 10 µl sample of each extract was placed in a well of a flat bottom 96-well plate (Becton Dickinson, Franklin Lakes, NJ), with each well containing 90 µl of deionized water. The plates included Pi standards consisting of proportionally diluting

K_2PO_4 in deionized water. Standards and samples were allowed to react with 100 µl of Chen's reagent (6 N sulfuric acid, 2.5% amomium molybdate, 10% abscorbic acid, deionized water) for 1 h, and absorbance at 882 nm was measured in a plate reader (Biotek, Winooski, VT). Samples were run in batches of 96 samples, and all the samples were run with the same batch of reagents. A sample of CX1834-1-6 (high Pi/low phytate) and Osage (low Pi/normal phytate) were also included in each batch of samples as controls for the extraction process. Data were presented in micrograms of Pi per gram of seed (μg^{-1}) and on an "as is" basis.

Statistical analysis

Analysis of variance was performed in the $F_{2:6}$ population considering line and location as random as fixed effects, respectively, and using the PROC MIXED procedure in SAS (Littell et al., 2006). Threshold values to separate phenotypic classes for the F_2-derived population were based on the present study, on previously reported Pi and sugar concentrations of the parental genotypes (Florez-Palacios, 2009; Mozzoni, 2009), and considering the shape of the population distribution (Mozzoni, 2009). The least significant difference (LSD) at 0.05 probability was calculated by multiplying standard error of the least square means by the table t-value at $p = 0.05$ (Bellaloui et al., 2009). Broad-sense heritability was calculated as described in Jaureguy et al. (2011). Correlations coefficients for the 2009 and 2010 data were estimated for each pair-wise combination of traits with the PROC CORR procedure in SAS (SAS Institute, 2002) using the breeding line least square means at each location. Yield stability was estimated as previously described in Maupin et al. (2011b) and Finlay and Wilkinson (1963). Briefly, yield data were log10 transformed, and the average yield of each breeding line was regressed on the average yield of all the breeding lines at each environment (environmental index). A desirable breeding line was defined as one with high yield, a stability coefficient (slope) around 1, and a high

coefficient of determination (less deviation from the regression), which would imply that the breeding line increased its yield as the productivity of the environment improved (Fehr, 1987).

RESULTS AND DISCUSSION

Analysis of variance and distributions of Pi, sucrose, and stachyose in the $F_{2:6}$ population

Inorganic phosphorus showed significant location effect, which indicated that the expression of this trait depended on the location where the lines were grown (Table 1). In contrast, sucrose and stachyose concentration were not significantly different between locations, which suggested that these traits were more environmentally stable than Pi, and that selection for *Mips* lines would not require growing the breeding lines in several environments.

Inorganic phosphorus and stachyose exhibited a bimodal distribution, while sucrose appeared to follow a normal distribution (Figure 1). Inorganic phosphorus distribution was skewed to the right towards the high Pi end with most individuals having low Pi, whereas stachyose showed a well-defined bimodal distribution with the majority of individuals having normal stachyose concentration. These results and the fact that the distribution fitted a 3 (normal phenotype with regular Pi and stachyose) to 1 (V99-5089 phenotype with high Pi and low stachyose) ratio (χ^2 = 1.04, p = 0.3), as in previous studies (Huhn, 2003; Florez-Palacios, 2009) confirmed that the low phytate and low stachyose traits in V99-5089 are controlled by a single recessive gene. The near normal distribution of sucrose concentration appeared to indicate that this trait is a quantitative trait conditioned by multiple genes with small effects. Quantitative trait loci for seed sucrose concentration have been reported in several genetic backgrounds and seem to be well spread across the soybean genome (Maughan et al., 2000; Feng et al., 2005; Kim et al., 2005; Kim et al., 2006). The skewness of Pi distribution, on the other hand, may be explained by the instability of Pi in V99-5089 derived germplasm (Maupin et al., 2011b; Jaureguy, 2012) and the lack of

perfect correlation between Pi and phytate concentrations. Evidence for the latter has come from studies in which Pi distribution of breeding populations segregating for the *Mips* mutation have been shown to be skewed to the right towards the high Pi parent (Jaureguy, 2012), whereas phytate concentration has been shown to exhibit a well-defined bimodal distribution (Saghai Maroof et al., 2011) similar to the one observed for stachyose in this study. This difference in trait distribution suggests that phenotypic selection for the *Mips* mutation would probably be more accurate using phytate than using Pi. However, if individuals that exhibit values as high as or higher than the parental genotypes are selected the selection should be as effective, at least in some genetic backgrounds (Rosso et al., 2011).

Heritability and seed trait correlations of the $F_{2:6}$ population

Broad-sense heritabilities were relatively high for the Pi, sucrose, and stachyose concentrations, which indicated that large percentage of the phenotypic variance observed for these traits was explained by differences among lines (Table 1). Therefore, phenotypic selection using Pi, sucrose, or stachyose is expected to be effective in this population, especially for the high Pi and low stachyose traits, and breeders can perform selections even in the early generations. Correlations among the three traits were highly significant and similar between locations, except for sucrose vs. stachyose at Fayetteville (Table 2). Stachyose was negatively correlated with Pi and sucrose, while Pi was positively correlated with sucrose. Higher correlation coefficients between Pi and stachyose than between Pi and sucrose confirmed that for this population, and in absence of stachyose data, phenotypic selection for individuals carrying the *Mip*s mutation using Pi may be more efficient than using sucrose. Contrarily, using other V99-5089 derived population grown in Arkansas, Jaureguy et al. (2012) showed that Pi may be the least effective of the three traits to identify *Mips* mutants. The disagreement between these

studies suggests that sucrose and Pi may be more environmental sensitive than stachyose concentration and, that in some cases, the genetic background in which the *Mips* mutation is expressed may have a large impact on the phenotype. The logical approach would be selecting for low stachyose, which most likely will lead to identification of high Pi and high sucrose. Further, considering that when samples are analyzed for sugar concentration, both sucrose and stachyose data are obtained in the same chromatogram, stachyose data should be the first choice to use in selection.

Yield stability and seed trait correlations of selected lines

Overall, the two check cultivars performed better than all high Pi lines (Table 3). Breeding line R08-6023, the highest yielding line, exhibited an average yield of 2894 kg ha^{-1}, which was significantly lower than the check yield average and the lowest yield check. Unlike this study, Maupin et al. (2011) reported that the average yield of three selected *Mips* lines grown in six environments did not differ from the high-yield checks, or the lowest yield check in another four environments. The average yield for the three *Mips* lines in the Maupin et al. (2011) study was 3180 kg ha^{-1}, whereas in this study the average yield of the 16 lines was 2141 kg ha^{-1}. Studies of low phytate lines derived from CX1834 (Hulke et al., 2004; Oltmans et al., 2005; Spear and Fehr, 2007; Scaboo et al., 2009) have shown that the low phytate trait for CX1834 germplasm does not negatively affect yield. However, because the low phytate trait in CX1834 is governed by different genes than in V99-5089, those studies may not necessarily imply that the *Mips* mutation does not negatively affect yield. The Maupin et al. (2011) and this study are the only two that have reported yields of *Mips* lines. Additional work with lines grown in other environments or with different pedigrees should be conducted before defining the effects of the *Mips* mutation on grain yield. It should be pointed out that in this study the selected *Mips* lines

were compared with commercial checks. If high Pi lines had been compared with low Pi lines from the same population, then the conclusion that the *Mips* mutation does not have a negative impact on seed yield might have reached. However, the fact is that the low phytate lines generally had significantly or at least numerically lower yields than the commercial checks, which presents a challenge for breeders to fill the gap in future breeding efforts. It is also worth noting that both parents for the population used in this study are specialty lines with value-added traits, which might be the reason for relatively low yield potential for the derived lines. The combination of high oleic acid (S02-529) with high sucrose and low phytate and low stachyose (V99-5089) in the lines studied is still valuable, even if the yield potential is low.

Three breeding lines (R06-6009, R06-6013, R06-6016) showed a stability coefficient (*b*) significantly different than zero (Table 3). Breeding line R08-6023 showed the highest yield compared to all the other breeding lines and the check cultivars (Table 3), but R08-6023 showed a slope not significantly different from 0 ($p = 0.594$) and a relatively low R^2. These results suggested that R08-6023 may increase its yield as the productivity of the environment improves, but the change would not be as predictable as other lines with better fit to the regression (Figure 2). However, from the practical breeding selection point of view, R08-6023 is still one of the best lines of choice among the lines tested (Table 3). The yield of R08-6023 was 81% of the overall check average, and did not differ from the check average in two of the five environments studied. R08-6023 can be likely released as cultivar or germplasm; it certainly will serve as an excellent parent for the next cycle of breeding for improved yield with low phytate and low stachyose. Further, data from the 2010 yield trials showed that parental genotypes of the selected lines, S02-529 and V99-5089, exhibited yields of 2266 and 435 kg $ha^{-1,}$ respectively, which shows the improvement of the selected lines over both parents.

On the other hand, R08-6009 ranked fourth in yield, exhibited a regression coefficient of 1.01 and $R^2 = 0.86$ (Figure 2), which suggested that R08-6009 could be used to produce low phytate /low stachyose in a variety of environments in Arkansas, with greater yields in highly productive environments and without considerable yield fluctuations. The yield of R08-6009 did not differ from that of R08-6023 in three of the five environments studied, and R08-6009 was considered, overall, the best line of the 16 evaluated in this study. Considering how yield is affected by factors such as weather and soil properties, having a cultivar with average yield in a wide range of environments (e.g. R08-6009) may be, sometimes, a better option to the cultivar whose yield is maximized only under specific conditions (i.e. R08-6023). However, lines with high stability should still be crossed to high yielding lines to produce improved high Pi/low stachyose progeny.

Most of the selected lines exhibited average protein, oil, and sats concentrations, and different than the levels expected to be considered as high protein (>45 mg g^{-1}), high oil (>22 mg g^{-1}) or low sats (<10 mg g^{-1}) lines (Table 4). However, some of the selected lines exhibited elevated oleic acid and reduced linolenic acid concentrations, which are desirable for oil crushing and production of value-added meal. Breeding line R08-6006 had moderately high oleic (458 mg g^{-1}) acid and three other lines (R08-6019, R08-6021, R08-6027) showed moderately low (<35 mg g^{-1}) linolenic acid concentrations. However, the yield and yield stability of these four lines were well below the average. Nevertheless, the improved fatty acid profile along with low phytate and low stachyose make these lines valuable as germplasm for release and/or parents for crossing in any breeding program. As expected and due to the selection imposed, all breeding lines exhibited high Pi, high sucrose, and low stachyose concentration. Breeding lines R08-6004 exhibited the highest sucrose concentration (88 mg g^{-1}) and R08-6037 exhibited the lowest (73

mg g^{-1}), all well above the commercial checks and a moderately significant improvement over V99-5089 (two Arkansas location average in 2010 = 72 mg g^{-1}). Breeding line R08-6023, the line with the highest yield, had a sucrose concentration of 81 mg g^{-1}, an stachyose concentration of 1 mg g^{-1}, and also moderately large seed size (19.9 g/100 seeds), which is also a very valuable trait in soybeans destined to the soyfood industry. Such lines with desirable seed quality traits and reasonable yields can be used for commercial production for the niche market where a premium is paid for quality traits and relatively low yields are more tolerated.

Yield, stachyose and Pi were not correlated with any of the other seed traits (Table 5), which suggested that the variation in yield, stachyose, and Pi among the selected lines was not large enough to generate significant covariation and consequently correlation among these traits. It is also likely that those additional quality traits (protein, oil, fatty acids) are, in these lines, independent of the targeted traits (Pi and stachyose) and yield, suggesting that selecting for those additional quality traits will not have an adverse effect on the targeted seed traits and yield, thus allowing breeders to combine all desirable seed traits into high-yielding backgrounds. Protein and oil concentration were negatively correlated with both sats and sucrose, which were positively correlated to each other. These correlations indicated that selecting for high protein in this germplasm may result in increased oleic acid, lower sats, and lower sucrose concentrations, the latter being the only response that would not be favorable. The extent to which selection for high protein may result in those responses deserves further investigation, as the variation for protein concentration in the lines studied was somewhat limited. Significant correlations do not imply that the variation that caused the correlation is enough to yield lines whose quality traits are equal or better than what is normally competitive and required by the market. Because the breeding lines evaluated in this study were not a random selection from a segregating population

as in most previous studies, the correlation patterns may have been affected and led to disagreements with some previously well-documented negative correlations in soybean seed traits, such as protein vs yield (Wilcox and Shibles, 2001; Wilson, 2004), protein vs oil (Geater and Fehr, 2000; Geater et al., 2000; Wilcox and Shibles, 2001; Chung et al., 2003; Wilson, 2004), and protein vs total sugars (Geater and Fehr, 2000; Geater et al., 2000; Wilcox and Shibles, 2001), all of which present a challenge for breeders to select against the unfavorable linkage between traits. However, using the *Mips* mutant would allow for moderate levels of protein along with high sucrose and low stachyose because of the favorable linkage between sucrose and stachyose despite the negative correlation between total sugar and protein concentration.

In this study, high Pi, high sucrose, low stachyose lines with stable yield potential for Arkansas growing environments were selected and identified, which represents a significant contribution for future development and commercialization of environmentally friendly soybean lines with increased nutritional value. Lines such as R08-6009 should continue to be tested in Arkansas and other soybean growing areas in the southern US and crossed to high yielding lines in order to obtain an improved yield stability assessment and increase the yield to competitive levels, respectively. Low phytate, high sucrose, low stachyose lines with low linolenic acid or high oleic acid, although relatively low in yield potential as compared to the commercial checks, could also be incorporated in breeding programs as a source of those trait combinations. These multiple-stacks would be of particular value for commercialization when they are incorporated into high-yielding backgrounds.

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Table 1. Analysis of variance of Pi, sucrose, and stachyose concentrations in127 $F_{2:6}$ lines derived from the cross S02-529 x V99-5089 grown at two Arkansas locations (Fayetteville and Keiser) in 2009, and trait broad-sense heritability for these seed quality traits.

Sources of effects	Dfn†	Dfd‡	§Pi		Sucrose		Stachyose	
			P value	MS¶	P value	MS	P value	MS
Location (loc)	1	2	0.037	412053	0.935	0.4	0.671	0.7
Heritability			0.96		0.86		0.96	

† degrees of freedom of the F test numerator.

‡ degrees of freedom of the F test denominator.

§ Pi = inorganic phosphorus (µg g-1), Suc = sucrose (mg g^{-1}), Sta = stachyose (mg g^{-1}).

¶ MS = mean squares

Table 2. Correlation coefficients among inorganic phosphorus (Pi), sucrose, and stachyose concentrations for 127 $F_{2:6}$ lines derived from the cross S02-529 x V99-5089 grown at Fayetteville, AR (above diagonal) and Keiser, AR (below diagonal) in 2009.

Trait	Pi	Sucrose	Stachyose
Pi		0.81***	-0.93***
Sucrose	0.85***		-0.76***
Stachyose	-0.95***	-0.84***	

*** significant at the 0.001 probability level.

Table 3. Least square means for yield and yield stability of 16 high Pi/low stachyose breeding lines selected from a $F_{2:6}$ population derived from the cross S02-529 x V99-5089, and grown in two (Keiser and Stuttgart) in 2010 and three Arkansas locations (Pine Tree, Stuttgart, and Rohwer) in 2011.

	Overall yield and descriptive statistics†						**Yield by environment‡**					**Stability parameters§**		
Breeding line	Yield	% CK	CV	SD	Max	Min	2010 KEI	2010 STU	2011 PTR	2011 ROH	2011 STU	*b*	R^2	*p*
CK56¶	3800		13	498	4601	2876	3792	2936	4094	4184	3993	0.55	0.44	0.22
CK49¶	3351		22	727	4502	2317	2379	2763	3887	3483	4244	0.97	0.47	0.20
R08-6023	2894	81	20	578	3681	2066	3427	2532	2760	2273	3476	0.35	0.10	0.59
R08-6009	2634	74	22	592	3876	1753	2624	2209	3332	2154	2851	1.01	0.86	0.02
R08-6011	2620	73	23	601	3570	1733	2099	2956	3084	2027	2936	0.52	0.18	0.47
R08-6021	2618	73	13	349	2979	1923	2792	2516	2866	2114	2800	0.44	0.34	0.29
R08-6037	2539	71	23	584	3586	1780	2208	2344	3449	2198	2496	0.98	0.73	0.06
R08-6039	2521	71	27	700	3382	836	2495	1338	2923	2813	3035	1.47	0.53	0.15
R08-6018	2507	70	21	516	3179	1457	3108	1814	2839	2286	2490	0.76	0.39	0.25
R08-6016	2422	68	34	836	4235	1241	2606	1712	3677	1906	2211	1.58	0.75	0.05
R08-6013	2416	68	25	565	3555	1178	2422	1680	3641	2020	2319	1.58	0.90	0.01
R08-6033	2416	68	30	720	3873	1594	2488	1724	3599	2220	2049	1.35	0.67	0.08
R08-6027	2391	67	26	631	3055	713	2603	1583	2752	2218	2802	1.13	0.63	0.10
R08-6019	2382	67	31	758	3814	1306	1502	1978	3377	2059	2996	1.56	0.64	0.10
R08-6017	2236	63	27	623	3478	1163	1407	2215	3034	2315	2211	0.83	0.27	0.37
R08-6004	2233	62	28	651	3125	1353	1312	2268	3077	1784	2726	1.23	0.36	0.28
R08-6006	2145	60	21	470	2810	1336	2241	1368	2565	2200	2349	1.17	0.66	0.09
R08-6024	2005	56	32	670	3150	1138	1315	1767	2995	2336	1610	1.03	0.28	0.35
#LSD	220						526	526	483	453	456			

† Yield in kg ha^{-1}, CK% = percentage yield of the check average, CV = coefficient of variation, SD = standard deviation of the overall mean, Max = maximum yield (single plot) observed for the line, Min = minimum yield (single plot) observed for the line.

‡ 2010KEI = data from 2010 Keiser, 2010STU = data from 2010 Stuttgart, 2011PTR = data from 2011 Pine Tree, 2011ROH = data from 2011 Rohwer, 2011STU = data from 2011 Stuttgart.

§ *b* = Finlay-Wilkinson regression coefficient for stability of yield (Maupin et al., 2011b), R^2 = coefficient of determination for the regression. A p value below 0.05 indicated that the regression coefficient was significantly different than 0 at the 0.05 probability level. A desirable breeding line was defined as one with high yield, a regression coefficient around 1, and a high coefficient of determination (less deviation from the regression), which would imply that the breeding line increased its yield as the productivity of the environment improved (Fehr, 1987).

¶ CK56 = check cultivar of relative maturity 5.6. 5601T was used in 2010, and AG5605 was used in 2011.

CK49 = check cultivar of relative maturity 4.9. UA 4910 was used in 2010, and AG4907 was used in 2011.

least significant difference at the 0.05 probability level.

Table 4. Seed quality traits of 16 high Pi/low stachyose breeding lines selected from a $F_{2:6}$ populations derived from the cross S02-529 x V99-5089 evaluated in two (Keiser and Stuttgart) in 2010 and three Arkansas locations (Pine Tree, Stuttgart, and Rohwer) in 2011.

Breeding line	Pro‡§	Oil§	Ole¶	Lin¶	Sats¶	Suc§	Sta§	Pi§	SS#	Qua#
CK56†	395	198	236	66	133	38	33	273	15.6	1.8
CK49†	369	214	264	57	141	45	38	255	15.9	2.8
R08-6023	407	191	328	46	138	81	1	1203	19.9	2.8
R08-6009	401	187	358	53	161	81	3	1298	20.1	2.3
R08-6011	402	184	269	51	161	76	4	1322	17.2	2.5
R08-6021	401	187	341	34	152	75	9	971	18.5	2.3
R08-6037	398	192	327	46	147	73	9	931	17.5	2.3
R08-6039	407	197	300	57	146	77	3	1436	19.2	2.0
R08-6018	403	188	324	52	146	78	4	1327	16.6	2.8
R08-6016	408	179	310	35	166	87	2	1183	18.1	3.0
R08-6013	412	188	376	36	144	76	9	1144	18.7	2.5
R08-6033	406	185	337	45	147	82	4	1391	17.7	3.0
R08-6027	403	186	294	34	143	80	4	1107	16.8	2.5
R08-6019	393	181	305	34	161	84	2	1203	17.2	2.8
R08-6017	410	194	302	60	135	73	3	1122	20.9	2.5
R08-6004	396	181	303	52	168	88	2	1293	16.8	3.0
R08-6006	424	184	458	41	132	77	3	1333	19.5	2.8
R08-6024	404	185	322	45	156	78	5	955	18.1	2.5

†CK56 = check cultivar of relative maturity 5.6. 5601T was used in 2010, and AG5605 was used in 2011. CK49 = Check cultivar of relative maturity 4.9. UA 4910 was used in 2010, and AG4907 was used in 2011.

‡ Pro = protein concentration (mg g^{-1}); Oil concentration in mg g^{-1}; Ole = oleic acid (mg g^{-1}); Lin = linolenic acid concentration (mg g^{-1}); Sats = saturated fatty acids (palmitic + stearic acids) (mg g-1); Suc = sucrose concentration (mg g^{-1}); Sta = stachyose concentration (mg g^{-1}); SS = seed size (weight of 100 seeds in grams); Qua = seed quality, rating from 1 (no split seed, no disease, very good overall appearance) to 5 (splits, disease on seeds, bad overall appearance).

§ one block per location in 2010 averaged with one block from Pine Tree in 2011.

¶ one block per location in 2010.

one block from both Stuttgart and Pine Tree.

Table 5. Correlation coefficients among yield and seed traits for 16 high Pi/low stachyose lines derived from the cross S02-529 x V99-5089 and grown at Keiser, AR and Stuttgart, AR in 2010.

Trait†	**Pro**	**Oil**	**Ole**	**Lin**	**Sats**	**Suc**	**Sta**	**Pi**
Yld	NS	NS	NS	NS	NS	NS	NS	NS
Pro		NS	0.63**	NS	-0.69**	-0.67*	NS	NS
Oil			NS	NS	-0.70**	-0.65**	NS	NS
Ole				NS	NS	NS	NS	NS
Lin					NS	NS	NS	NS
Sats						0.77***	NS	NS
Suc							NS	NS
Sta								NS

†Yld = grain yield (kg ha^{-1}); Pro = protein concentration (mg g^{-1}); Oil concentration in mg g^{-1}; Ole = oleic acid concentration (mg g^{-1}); Lin = linolenic acid concentration (mg g^{-1}); Sats = saturated fatty acids (palmitic + stearic acids) (mg g-1); Suc = sucrose concentration (mg g^{-1}); Sta = stachyose concentration (mg g^{-1}); Pi = inorganic phosphorus concentration (µg g^{-1}).

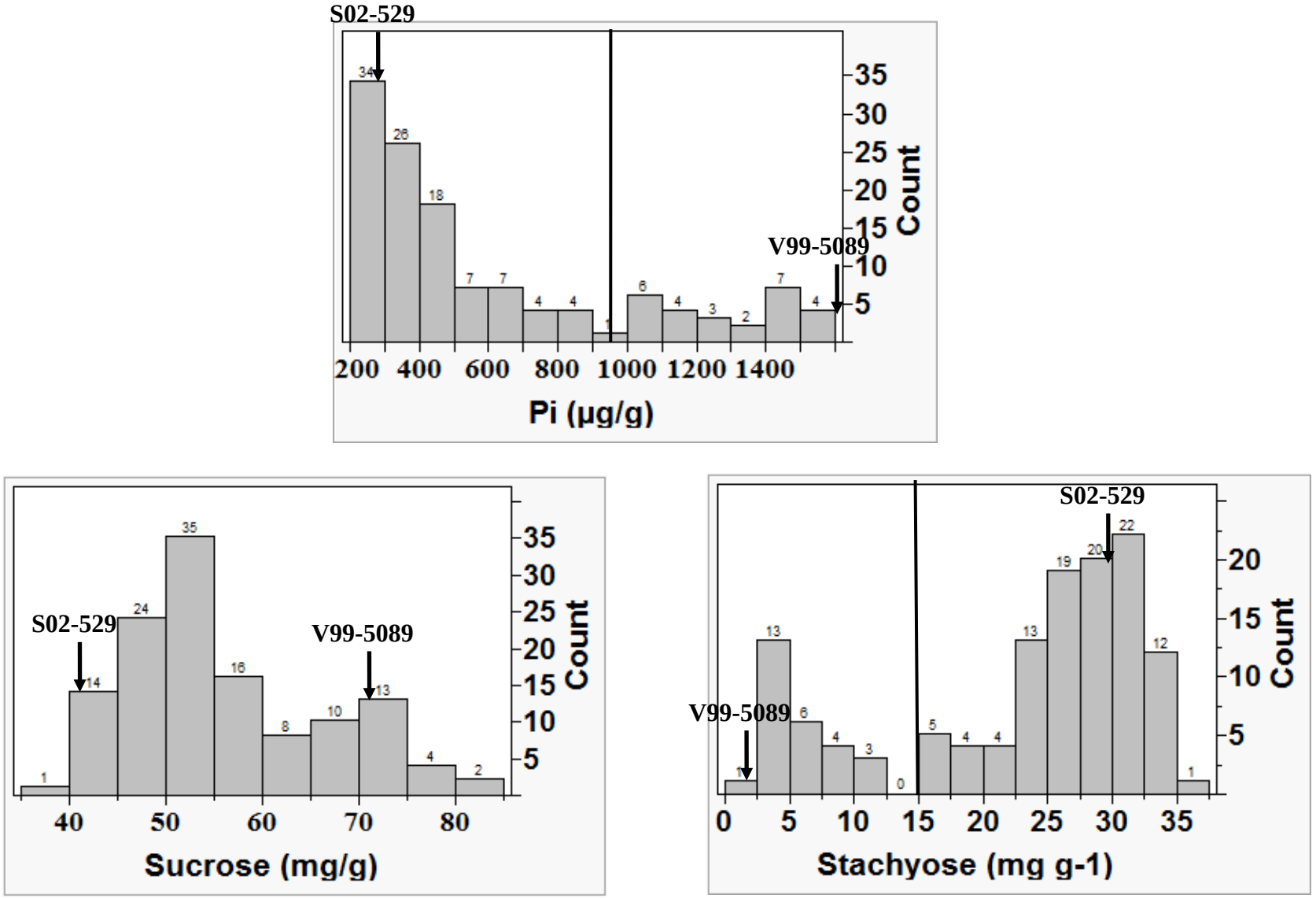
NS, not significant at the 0.05 probability level.

* significant at 0.05 level.

** significant at 0.01 level.

*** significant at 0.001 level.

Figure 1. Distribution of Pi, sucrose, and stachyose concentrations in127 $F_{2:6}$ lines derived from the cross S02-529 x V99-5089 grown at two (Fayetteville and Keiser) Arkansas locations in 2009.

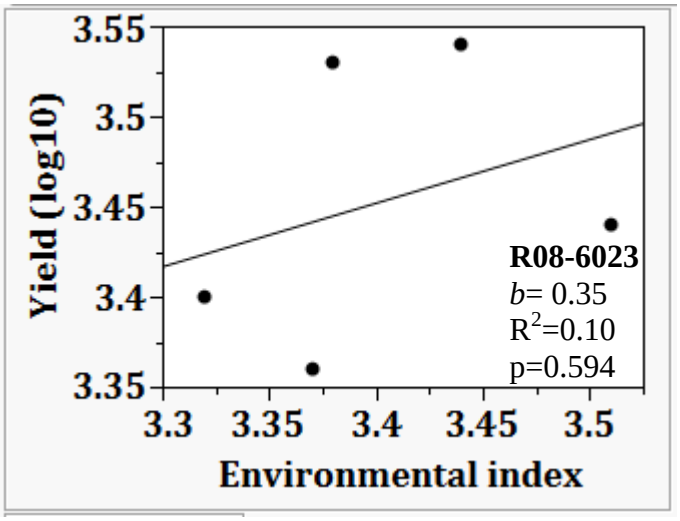


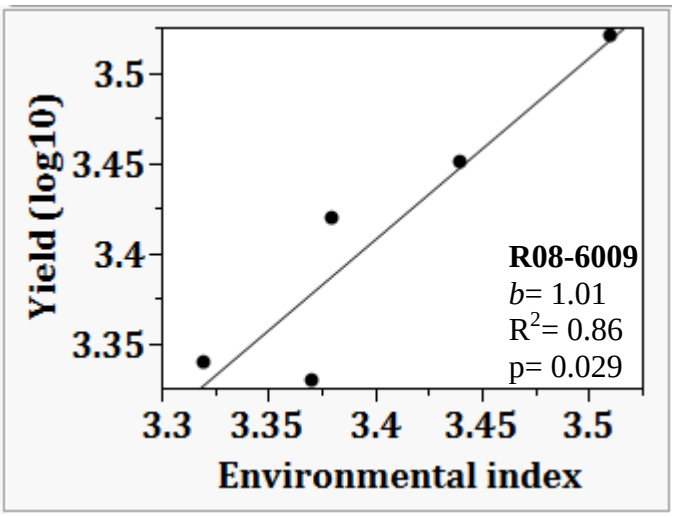
Arrows in graphs indicate the mean value of the parental genotypes S02-529 and V99-5089 for each trait measured. S02-529 mean ± 2 standard deviations (STD) for Pi (203-301 µg g^{-1}), sucrose (28-55 mg g^{-1}) and stachyose (9-45 mg g^{-1}). V99-5089 mean ± 2 STD for Pi (918-2029 µg g^{-1}), sucrose (55-89 mg g^{-1}) and stachyose (0-4 mg g^{-1}).

Vertical line in Pi and stachyose graphs separates the two phenotypic classes: those that exhibited V99-5089-like phenotypes from those that exhibited normal levels of Pi and stachyose. Threshold values to separate phenotypic classes for the F_2-derived population were based on the present study, previously reported Pi and sugar concentration of the parental genotypes (Florez-Palacios, 2009; Mozzoni, 2009; Jaureguy, 2012), and considering the shape of the population distribution (Mozzoni, 2009).

Chi-square tests showed that high Pi/low stachyose concentrations fitted a 3 (normal) : 1 (high Pi/low stachyose) ratio ($\chi^2 = 1.04$, $p = 0.3$), as in previous studies (Huhn, 2003; Florez-Palacios, 2009).

Figure 2. Regression of log10 transformed yield per environment on environmental index (average yield of all the breeding lines at each environment) for high Pi/low stachyose breeding lines R08-6023 and R08-6009 grown in two (Keiser and Stuttgart) in 2010 and three Arkansas locations (Pine Tree, Stuttgart, and Rohwer) in 2011.





IV. Planting date and delayed harvest effects on soybean seed composition

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Abbreviations: int, intermediate planting date; HPLC, high performance liquid chromatography; Kib, Kibler, AR; Fay, Fayetteville, AR; dfn, degrees of freedom of numerator; dfd, degrees of freedom of denominator; LSD, least significant difference; Pro, protein; Ole, oleic acid; Lin, linolenic acid; Sats, saturated fatty acids; Suc, sucrose; Sta, stachyose; Pi, inorganic phosphorus; P, phosphorus; K, potassium; Ca, calcium; Mg, magnesium; S, sulfur; Fe, iron; Mn, manganese; Zn, zinc; Cu, copper.

ABSTRACT

Information on crop management practices that may help maximize farm profit is valuable. Because planting date determines the environment to which the crop is exposed during the growing season, it can also have a significant impact on seed composition. In this study, the effects of planting date on seed composition were investigated using eight specialty soybean breeding lines with modified seed composition grown at two Arkansas locations for two years. The effects of delayed harvest on seed composition were also studied in one of the planting dates. Seed compositional traits included protein, oil, sucrose, stachyose, inorganic phosphorus, oleic acid, linolenic acid, saturated fats, and nine chemical elements including phosphorus, potassium, calcium, magnesium, sulfur, iron, manganese, zinc, and copper. Planting date significantly affected seed organic and inorganic composition. Early planting increased seed protein, oleic acid, and inorganic components, and decreased linolenic acid concentrations, whereas late planting increased sucrose, but did not affect stachyose concentrations. Production of soybeans with high protein/high quality oil should be performed in early-planting production systems, whereas planting high sugar food-grade cultivars late in the season should fit well in a double-crop system. Lower temperatures experienced by late-planted soybeans during the seed-fill period may have been responsible for the observed results. Delayed harvest had statistically significant, but small, effects on both organic and inorganic seed components. Seed components exhibited lower concentration in soybeans that experienced delayed harvest. Unlike with planting date, the effects of delayed harvest were independent of breeding lines. These research findings may help enhance recommendations to farmers to adjust planting and harvest practices and consequently meet market requirements for specialty soybeans.

INTRODUCTION

Specialty soybeans have value-added seed traits and are targeted for niche markets. Examples of specialty soybean include food-grade soybeans, which have improved seed composition (high protein, high sucrose, and low stachyose concentrations) for enhancing nutritional value and digestibility, modified oil soybeans, which contain high oil concentration and healthier and more desirable fatty acid profiles [(high oleic acid, low linolenic acid, and low saturated fatty acids (sats) concentrations], and environmental friendly soybeans, which contain low phytate concentration for decreasing the amount of phosphorus (P) in animal manure.

High protein concentration has been shown to be correlated with tofu yield and firmness (Poysa and Woodrow, 2002). Further, soybeans with high protein concentration produce soybean meal with increased nutritional value for soybean-based animal feed. High sucrose and low stachyose improve soyfood flavor and digestibility (Mebrahtu and Devine, 2009). High oleic acid and low linolenic acid make soybean oil healthier (higher concentration of monounsaturated fats, lower concentration of polyunsaturated fats), and improves its oxidative and heat stability, which is more attractive for frying and edible applications. Low saturated fats (stearic and palmitic acid) concentration also contributes to oil quality and flavor stability, and helps reduce the risk of coronary disease (Simopoulos, 1999). Low phytate soybeans results in high inorganic phosphorus (Pi) and nutritionally efficient animal feed. As a result, more P is available to be consumed and incorporated by the animal and less is excreted. Low P manure helps ameliorate the risk of eutrophication of waterways caused by increased amount of nutrients in farmland runoff water.

Despite their importance in global human health and the presence of genetic variation (Raboy et al., 1984; White and Broadley, 2009), seed chemical elements have not been, compared to

other traits, a common target for breeders developing specialty soybean cultivars. A major impact of these types of cultivar would be on animal diets and in the soyfood production industry. Increased amount of chemical elements combined with low concentration of phytate (chelates chemical elements in the seed) would render soybean meal with greater nutritional value for animal feed production. Similarly, modified chemical element concentration (e.g. low Ca) would help improve the quality of soyfood (e.g. tofu) and make whole soybean foods (e.g. edamame) more nutritious. Understanding how chemical elements respond to environmental variables and management practices would help develop breeding strategies for specialty cultivars with modified inorganic seed composition in the future.

Although the primary objective of growing a crop is to maximize yield (Graham et al., 1999) and obtain the highest profit, seed quality and composition may become as important in the case of specialty soybeans. The extent to which seed quality parameters are met by the crop will determine the value of specialty soybeans. Farmers growing specialty soybean should not only choose cultivars that have been bred for specific quality traits, but also manage the crop properly in order to optimize the expression of these traits. Because cultivars respond differently to the environment and agricultural practices, information on what management practices that can help with this endeavor should be valuable (Rengel et al., 1999; Gao et al., 2009). A practical approach to this would be to study these cultural practices in breeding lines with various targeted traits and that are in a breeding program's release pipeline, and then have the information delivered to the farmer when the cultivar is released.

Planting date is a management practice that can have major effects on the crop's overall growth and development (Anderson and Vasilas, 1985; Pedersen and Lauer, 2004; De Bruin et al., 2010). The range of temperature, soil moisture levels and day length to which soybeans are

exposed during the growing season, within the same location, largely depends on planting date. Because chemical and enzymatic reactions are temperature sensitive, biochemical processes involved in the synthesis and remobilization of seed components may therefore be affected by temperature. Research has showed that organic and inorganic seed components are significantly affected by temperature during the reproductive period in soybean (Gibson and Mullen, 2001; Ren et al., 2009) and other crops (Fenner, 1992). In addition, changes in moisture levels can cause plants to perform osmotic adjustments that result in chemical element translocation, which can result in significant changes in seed composition (Samarah et al., 2004). For example, drought-stressed soybeans were shown to have higher seed protein concentration than non-stressed ones under laboratory conditions (Pikaard and Cherry, 1984). Day length affects the timing of transition from the vegetative to reproductive stage and consequently the amount of dry matter destined to each of the two growth stages. This will affect the amount of carbon that can be assimilated by the crop and how it will be partitioned among plant parts (Cure et al., 1982), the length of the seed-fill period (Thomas and Raper Jr., 1976) and possibly seed composition (Howell and Collins, 1957).

Although the causal basis is still unknown (Wilson, 2004), previous research has shown that, of the three factors described above, temperature during seed development is the primary environmental factor governing seed composition (Feaster, 1949; Weiss et al., 1952; Wolf et al., 1982; Kane et al., 1997; Piper and Boote, 1999). A significant portion of the literature on this topic of research consists of field studies in which an association between seed composition of a group of cultivars and temperatures from weather stations was evaluated. The general consensus is that, for the same maturity group soybeans, the later the soybean is planted, the more likely the seed-fill period takes place in cooler temperatures than early-planted soybeans. This results in

that early planting is usually associated with an increase in oil and oleic acid, and a decrease in sugars, linoleic and linolenic acids concentrations, and that saturated fatty acid shows the least response to changes in planting date or temperature of seed development.

The timing of harvest is another factor in crop management that can have significant effects on seed quality (Wilcox et al., 1974; Woodstock et al., 1985) and composition (Krober and Collins, 1948; Woodstock et al., 1985; Yaklich, 1985). Farmers are sometimes confronted with adverse conditions (weather, machinery failure, etc.) that prevent them from harvesting their crop before yield losses occur (Philbrook and Oplinger, 1989) or diseases (Wilcox et al., 1974; Larcher, 1985; Dao and Ram, 1996) and weather deteriorates seed quality. For example, 2009 had an extremely wet fall in the major US soybean growing areas. The USDA reported that the 2009 harvest was, at that time, the slowest ever, with only 46% of the soybean area harvested by October 25th. In 2008, 76% of the soybeans had been harvested by that date (Theisse, 2009).

There are only a few publications on the effect of delayed harvest on seed composition and probably none on the seed inorganic composition changes with delayed harvest. Krober and Collins (1948) compared the seed composition between good quality and weather-damaged soybeans. Delayed harvest and exposure to adverse weather caused an increase in crude protein, decrease in sugars, and no significant effect on oil concentrations. Yaklich (1985) studied the effects of delayed harvest on seed sugars (sucrose, raffinose, stachyose) in six cultivars grown in Maryland during three consecutive years. Seed of these cultivars was harvested at maturity and again after about six weeks. Delayed harvest had no significant effect on seed sugars. Further, the difference between soybeans harvested at maturity and that experienced delayed harvest were small. Adak et al. (2007) harvested chickpeas (*Cicer arietinum*) at three different times and observed that protein, P, calcium (Ca), magnesium (Mg), copper (Cu), zing (Zn), and manganese

(Mn) concentrations were greater at optimum harvest time than at either early or late harvest times. The authors proposed that the reason for the observed differences was that the time elapsed among harvest dates allowed for translocation of nutrients between vegetative and reproductive (seeds) part of the plants (Adak et al., 2007).

The hypotheses of this study were that planting date and harvest time will affect the organic and inorganic composition of soybeans seeds, as the crop will experience different environments during the growing season (e.g. temperature, moisture) with different planting dates.

The objectives of this study were to evaluate the effect of planting date on the seed composition in four types of specialty soybeans: high protein, high oil, modified fatty acids, and low phytate/low stachyose, grown two years at two Arkansas locations. As a secondary objective, the effects of delayed harvest on seed composition were studied, but only on one of the planting dates. Lines used were the best quality trait lines from the University of Arkansas' soybean breeding program. Some of them have been or will be released as germplasm with enhanced seed composition quality in the future. Further, these specialty soybean breeding lines have been crossed to high-yielding lines and are represented in the program's germplasm. Understanding the timing of planting and harvest of specialty soybeans may help producers develop efficient management strategies to meet market quality demands and consequently maximize crop profit.

MATERIALS AND METHODS

Field experiment

Eight breeding lines with modified seed composition and a high-yield check were used for this study (Table 1). The experiment was arranged in a split-plot with three blocks, with planting date as the main plot, arranged in randomized complete block design, and breeding line as the

subplot. The whole plot consisted of a field strip containing 10 subplots. Each subplot consisted of a 5-m four-row plot. Seeding rate was 30 seeds per meter. The experiment was planted at the Arkansas Agricultural Research and Extension Center in Fayetteville, AR, and at the University of Arkansas Vegetable Research Station in Kibler, AR. The soil at the Fayetteville location in 2010 is mapped as Captina silt loam (fine-silty, siliceous, active, mesic Typic Fragiudult) (Soil Survey Staff, 2011) and described as very deep, moderately well-drained soil developed on a thin mantle of silty material. There was no crop in that field the previous year. The soil in Fayetteville in 2011 is mapped as Leaf silt loam (fine, mixed, active, thermic Typic Albaquults)(Soil Survey Staff, 2011). The previous crop in that field was wheat (*Triticum aestivum*). At Kibler, the soil is mapped as Roxana silt loam (coarse-silty, mixed, superactive, nonacid, thermic Typic Udifluvents) (Soil Survey Staff, 2011) and is described as a well-drained, moderately permeable soil formed in stratified loamy alluvium (Garner and Cox, 1979). The previous crop in these fields both in 2010 and 2011 was soybean. Row spacing was 100 cm and 90 cm in Fayetteville (planted on beds) and Kibler (planted on flat ground), respectively. Planting dates were early May (early), late May (intermediate) and late June (late). Planting dates in 2010 were May 6th, May 29th, and June 22nd in Fayetteville and May 5th, May 28th, and June 24th in Kibler. Planting dates in 2011 were May17th, June 2nd, and June 21st for Fayetteville and May 10th, May 31st, and June 21th for Kibler. Field plots were fully irrigated (furrow irrigation at Fayetteville; overhead at Kibler) and managed during the growing season using standard cultural practices adopted for full- season soybean production in Arkansas (Tacker and Vories, 1998). Cultural practices included tillage with chisel plow and disc, and fertilization based on soil test results and recommendation of University of Arkansas Cooperative Extension Service. Weed control was performed by applying pre-plant and post-emergence herbicides (e.g. glyphosate), at label rates.

At maturity, the two center rows of the plots were harvested using a plot combine. Seed was stored in cloth bags at room temperature until seed composition analysis.

Delayed harvest

The effects of delayed harvest on seed composition were studied only in the intermediate planting date which is typical for full-season commercial soybean production in Arkansas. Although the breeding lines used in this study belonged to the same maturity group, some lines matured slightly earlier than others, but within a narrow range. To account for those differences, plots were monitored periodically and the two center rows were harvested as soon as they reached physiological maturity (95% of the plots showed mature color). About 14 to 20 d later, a portion of the border rows of each plot was harvested as the delayed harvest. Comparison of seed harvested at maturity and seed harvested 14 to 20 d later was used to determine the effects of delayed harvest on seed composition.

Seed composition analysis

Protein and oil analysis. Samples containing 20-25 g of seed were sent to the USDA research facility at Peoria, IL or to the University of Missouri Delta Center at Portageville, MO for protein, oil and moisture analysis in a FOSS® (Eden Prairie, MN) near infrared transmittance instrument. Light of specific wave length interacts with the sample's organic compounds (e.g. protein and oil), which then transmit different light intensity depending on their concentration in the sample. Protein and oil concentration were presented in milligrams per gram of seed (mg g^{-1}) and in dry-weight basis.

Fatty acid analysis. Samples containing five seeds were sent the DNA facility at Iowa State University in Ames, IA. Fatty acid concentrations [oleic acid, linolenic acid, and saturated fatty acids (sats)] were determined by gas chromatography according to the methods developed by

Hammond (1991)**.** The fatty acid concentration was presented in milligrams per total grams of oil (mg g^{-1}) and on an "as is" basis.

Sugar analysis. Each seed sample was processed separately for sugar extraction, sugar fractionation and identification, and sugar quantification by high-performance liquid chromatography (HPLC). For a detailed description on the HPLC methods see Jaureguy et al. (2012). Sugar data were presented in milligrams of sugar per gram of seed (mg g^{-1}) and on an "as is" basis.

Inorganic chemical element analysis. A 10 g seed sample was ground in a coffee grinder (Krups®, Shelton, CT) and the powder was screened through a 450 μm sieve (VWR International, West Chester, PA). Samples were stored in air-tight 7.5 x 10 cm plastic bags (Uline, Dallas, TX) and submitted to the University of Arkansas Soil Testing and Research Laboratory in Fayetteville, AR for seed inorganic composition. Samples were digested using concentrated HNO_3 and 30% hydrogen peroxide on a heating block system (Environmental Express, Mt. Pleasant, SC). Samples were analyzed using Spectro Arcos Inducted Coupled Plasma Mass Spectrometer (Spectro Analytical Instruments, Mahwah, New Jersey). Data were presented as milligram of element per gram of seed (mg g^{-1}) for P, potassium (K), Ca, Mg, and sulfur (S), and as milligrams of element per kg of seed (mg kg^{-1}) for iron (Fe), Mn, zinc (Zn), and Cu, and on an "as is" basis.

Pi analysis. Inorganic phosphorus was measured to represent the phytate levels and was based on an inverse relationship between phytate and Pi that has been reported for lines derived from V99-5089 and other low phytate germplasm sources (Maupin et al., 2011a). A sample of 0.1 g of ground and sieved soybean seed fine powder was weighed in a 2.0 ml centrifuge tube. One ml of extraction buffer (deionized water, 12.5% trichloroacetic acid, 1 M $MgCl_2$) was

added, and the solution was vortexed until it became homogeneous. The solution was incubated overnight (≈16 h) at 4ºC. Then, the sample was vortexed, incubated at room temperature for 30 min, and centrifuged at 1400 *g* for 4 min. Samples were stored at 4ºC until Pi analysis. A 10 µl sample of each extract was placed in a well of a flat bottom 96-well plate (Becton Dickinson, Franklin Lakes, NJ), containing 90 µl of deionized water. The plates included Pi standards, consisting of proportionally diluting K_2PO_4 in deionized water. Standards and samples were allowed to react with 100 µl of Chen's reagent (6 N sulphuric acid, 2.5% amomium molybdate, 10% abscorbic acid, deionized water) for exactly 1 h, and absorbance at 882 nm was measured in a plate reader (Biotek, Winooski, VT). Samples were run in batches of 96 samples, and all the samples were run with the same batch of reagents. A sample of CX1834-1-6 (high Pi/low phytate) and Osage (low Pi/normal phytate) were also included in each batch of samples as controls for the extraction process. Calculation of Pi concentration in samples was done by linear extrapolation using the formula µg Pi = area/curve slope. The conversion factor was calculated according to the changes in concentration that Pi undergoes during extraction procedures. Data were presented as micrograms of element per gram of seed (µg g^{-1}) and on an "as is" basis.

Weather data

Maximum and minimum daily air temperature and precipitation during the growing season were recorded by the University of Arkansas Experimental Stations where the experiment was conducted. Data were requested to the stations directors and processed to use in this study.

Statistical analysis

Planting date effects. The combinations of years and locations were considered as four different environments and included in the statistical model. The experiment was analyzed as a split-split plot with environment as the whole plot, planting date as the split-plot, and breeding

line as the split-split plot. All the factors were considered fixed in the analysis of organic seed composition. However, because breeding lines had not been bred for any specific chemical element, they were considered a random effect in the analysis of chemical elements data. Data were analyzed using the PROC MIXED procedure in SAS 9.2 (SAS Institute, 2002), which allowed to test main effects and interactions with the appropriate error term (Littell et al., 2006). Significant interactions were further investigated with the "slice" option in PROC MIXED, which allowed testing main effects or interactions at different levels of other main effects. Means were estimated and compared using the "lsmeans" and "contrast" options, respectively. The least significant difference (LSD) at 0.05 probability was calculated by multiplying the standard error of the least square means by the table t-value at 0.05 (Bellaloui et al., 2009).

Delayed harvest effects. Because the delayed harvest experiment was conducted only on the intermediate planting date, a separate data analysis was performed. Data were analyzed as split-split plot with environment as the whole plot, line as the split-plot, and harvest time as the split-split plot. Data were analyzed using the PROC MIXED procedure in SAS (SAS Institute, 2002) and using the same options as used in the planting date analysis.

RESULTS AND DISCUSSION

Planting date effects on organic seed composition

The overall ANOVA showed that breeding line by environment interaction was significant on all the seed quality traits studied (Table 2). The significant genotypic variance was expected as lines were selected to represent large variations in those traits. Protein, linolenic acid, sucrose, stachyose, and Pi concentrations were significantly influenced by planting date (Table 2). Across planting dates and environment, high protein lines R05-1772 and R05-1415 exhibited higher protein concentration than the rest of the lines (R05-1415 vs. other lines = 31 mg g^{-1}, $p<0.001$;

R05-1772 vs. other lines = 25 mg g^{-1}, $p<0.001$) (Table 3, Figure 1). Both lines exhibited significantly lower protein concentration in the late planting (R05-1415 late vs. other planting dates = 9 mg g^{-1}, $p = 0.0017$; R05-1772 vs. other planting dates = 11 mg g^{-1}, $p<0.001$) and in year 2011. The decrease in protein was generally accompanied by an increase in sugar concentration. It was reported that high-yield lines have shown gradual decrease in protein when planted later than the second week of May in other studies with soybean in Arkansas and South Carolina (Beatty et al., 1982; Ray et al., 2008). As protein decreases with delayed planting, one would expect an increase in oil concentration because of their strong negative correlation (Geater and Fehr, 2000; Geater et al., 2000; Wilcox and Shibles, 2001; Chung et al., 2003; Wilson, 2004). However, oil concentration remained somewhat consistent across planting dates, thus the metabolism was perhaps devoted towards sugars instead of oil as a result of decreased protein with delayed planting. The two high-protein lines evaluated in this study would be good alternatives for Arkansas farmers who want to grow soybeans to be used in the production of high protein soybean meal. In this case, early planting is encouraged to maximize the seed protein levels.

The slice test showed that three lines, including the high oil line R02-6268F, showed no planting date by environment interaction for oil, indicating that their oil concentration was stable across environments (Table 2). The other high oil line R05-655, on the other hand, exhibited a significant planting date by environment interaction ($p<0.001$). R05-655 did not differ between planting dates across environments ($p = 0.37$), which suggested that the effects of the environments on oil concentration for this line were more important than the effect of planting dates, and contributed more to its low stability. Although both high oil lines showed significantly higher oil than the rest of the lines (R02-6268F vs others = 12 mg g^{-1}, $p<0.001$; R06-655 vs

others = 17 mg g^{-1}, $p<0.001$), only R06-655 at Kibler in 2010 and early plantings exhibited oil concentration (217 mg g^{-1}) close to the expected range (>220 mg g^{-1}) for a competitive high oil soybean line. These two lines had exhibited those values in previous testing at the University of Arkansas Experimental Stations (data not shown). Because of a lack of interaction between planting date and environment, and higher yield potential (see below), R02-6268F may be a better option than R06-655 to attain high oil in a variety of planting dates. Breeding line R02-6268F should be grown in locations that would increase oil concentration to levels accepted by market standards. More data on the plasticity of this line and its ability to attain high oil levels should shed light on whether this line has the competitive potential. Further, as previously reported, early planting should favor high oil concentration (Torrie and Briggs, 1955; Kane et al., 1997; Dardanelli et al., 2006; Ray et al., 2008; Robinson et al., 2009).

High oleic line R07-8292 exhibited significant planting date by environment interaction for oleic acid concentration (Table 3). Breeding line R07-8292 exhibited high oleic acid concentration in early planting and decreased significantly with later planting dates in 2010, but slightly in 2011 (Figure 1). Apparently, early planting allowed the seed-fill stage coincide with higher temperature which enhanced the oleic acid level, as also observed in other studies (Wilcox and Cavins, 1992; Kane et al., 1997; Wilson, 2004; Bellaloui et al., 2011). The oleic acid concentration of R07-8292 was, averaged across environments, always below the desired level (500 mg g^{-1}) (Table 3). These results suggested that oleic acid in R07-8292 was not a stable trait and, therefore, this line may not be an adequate option for farmers intending to maximize oleic acid in soybean oil. Carver et al. (1986) reported that lines that had been selected for high oleic acid were more sensitive to environmental variation than unselected lines, which agrees

with the differences that were observed between R07-8292 and the other lines evaluated in this study.

The linolenic acid concentration of line R05-5346 showed no planting date by environment interaction. The low linolenic trait seemed to be a rather stable trait as compared to the high-oleic trait. Although it showed an increasing significant trend with planting date (Table 3), linolenic acid concentration of breeding line R05-5346 was always low and within the desired range (<350 mg g^{-1}). Similar results have been observed in other cultivars with the same trait (Wilcox and Cavins, 1992), lines that have been selected for different fatty acids (Schnebly and Fehr, 1993; Ray et al., 2008), and high-yield lines of earlier maturities (Kane et al., 1997; Bellaloui et al., 2011). R05-5346 would be a good choice for farmers interested in producing soybeans with low linolenic acid and with flexible planting date.

Averaged across planting dates, breeding lines exhibited significantly different yields among environments. In the slice test all lines had significant differences in yield across environments ($p<0.001$). The check cultivar AG5605 was, as expected because it was bred for high yield, the only genotype that had significantly different yield than all the other genotypes in the multiple comparisons among breeding lines. Further, AG5605 showed the highest average yield across environments and planting dates (Table 3). All breeding lines, except R05-1772 (78%, 2465 kg ha^{-1}) , R06-814 (74%, 2020 kg ha^{-1}), and R07-8292 (56%, 1759 kg ha^{-1}), had, averaged across planting dates and environments, at least 85% of AG5605's average yield (3154 kg ha^{-1}), with R02-6268F having 88% (2790 kg ha^{-1}). These results suggested that, in terms of yield potential, R02-6268F and R05-1415, may be chosen over R06-655 and R05-1772 as high oil and high protein lines, respectively. Further, R07-2000 exhibited yields that were superior to other low phytate lines previously studied (Hulke et al., 2004).

For saturated fatty acids (sats), R06-814 was more stable among environments ($p = 0.08$) than planting dates ($p = 0.009$). R06-814 had significantly, but slightly, greater sats concentration for late than early planting dates in three of the four environments studied. However, mixed results were reported in other studies. Kane et al (1997) showed little or no effect of planting date on saturated fats in conventional cultivars (no quality traits), whereas Ray et al. (2008) observed little effect on palmitic acid concentration (early = 76 mg g^{-1}, late = 74 mg g^{-1}) in late-planted breeding lines with modified sats. Breeding line R06-814 was always below the desired limit (100 mg g^{-1}), which suggested that R06-814 maybe an adequate choice for the production of low sats oil in Arkansas.

All lines had significant differences in sucrose concentration among environments and among planting dates. Line R07-2000 exhibited significantly greater sucrose and Pi concentrations than the other lines, and sucrose and Pi of R07-2000 were never lower than the desired concentration (sucrose = 85 mg g^{-1}, Pi = 1600 µg g^{-1}). All lines, including R07-2000, had greater sucrose concentration at later plantings (Figure 1). It appears that the fact that lines had been selected for seed components other than sucrose, stachyose, or Pi did not have a significant impact on these lines' sucrose response to planting date. Genetic factors controlling the response of sucrose to environmental conditions are likely well conserved among the breeding lines studied. Thus, late planting dates will favor both commodity (high yield, not selected for seed quality traits) and modified sugar breeding lines for the production of high sucrose-low stachyose soybean meal. This is important in selecting an optimum planting date for the specialty soybeans for natto, tofu, edamame and soymilk, which require high sucrose and low stachyose concentration (Mebrahtu and Devine, 2009). Stachyose concentration had no planting date by breeding line interaction ($p = 0.51$)(Figure 1). Thus, R07-2000 could be planted up to the third

week of June without a significant change in stachyose and with up to 20 mg g^{-1} increase in sucrose. Little differences in Pi concentration among environments and planting dates suggested that R07-2000 had stable phytate concentration. The range of Pi observed for R07-2000 (1656 - 2340 µg g^{-1}) was well within the amount of Pi associated with low phytate genotypes with similar pedigree and carrying the same high Pi, high sucrose, low stachyose mutation (Maupin et al., 2011a; Maupin et al., 2011b; Rosso et al., 2011). The stability of low stachyose and phytate from this mutant source makes the management practice simple in commercial production of such special type soybeans.

Planting date effects on inorganic seed composition

All inorganic seed components showed a significant environment by planting date interaction (Table 4). Iron and Cu were the only two elements that, across environments, did not show significant differences among planting dates. The stability of seed Fe across planting dates and the fact that soybean is an excellent source of Fe for the human diet (Messina, 1999; Lukac et al., 2009) makes Fe a desirable breeding target. Potassium, Mg, Mn, were, but only slightly, lower with late planting date at all the environments, except when plants were grown at Kibler in 2010 (data not shown). Calcium consistently decreased from early to late planting dates in all four environments (Table 5, Figure 1). Because soybean seed Ca concentration is positively correlated with seed hardness (Zhang et al., 2009), which may affect the texture and quality of soyfoods such as tofu and edamame, late planting of cultivars destined to food production may contribute to meet these market standards.

Although numerical changes among planting dates were observed, seed chemical element concentrations were accompanied by numerous and little fluctuations that defined no clear pattern that could be used to make planting decisions.

Weather data and changes in seed composition

While considering the entire data set, two patterns of response of seed components to planting dates and environments were observed. All organic seed components, with exception of sucrose, linolenic acid, and sats, and most of the inorganic components, tended to decrease with delaying planting date. The same components that showed low values at late planting date also exhibited low concentration in 2011 compared to 2010. The similarity between late planting and year 2011 is that late-planted soybeans usually experience cooler temperatures than those planted early (Kane et al., 1997), and 2011 showed lower daily minimum temperatures than 2010 during month of September. Temperature data showed that the month of August in 2011 was 4.7°C, and 4.8°C warmer (maximum day temperature) than in 2010 in Fayetteville and Kibler, respectively (Table 10). However, 2011 exhibited, averaged across locations, lower daily minimum temperatures (12.9°C) than in 2010 (17.3°C) in the month of September, which coincides with a large portion of the seed-fill period of the lines (maturity group V) used in this experiment. Research has shown that protein concentration is generally high in southern states due to higher temperatures (Burton, 1987). Piper and Boote (1999) also showed an increase in protein and oil concentrations with increased air temperature in 20 cultivars grown during 20 years over an average of 60 locations in the northern US. Likewise, Ray et al. (2008) showed a significant decrease (4 mg g^{-1}) in both protein and oil concentration for late-planted modified fatty acid breeding lines. In Ray et al. (2008) experiment, soybeans seed-fill period took place in maximum daily temperatures that ranged from 27°C to 21°C in early-planted soybeans, and from 23°C to 20°C in late-planted soybeans, which, according to the authors, explained changes in seed protein with planting date. In another study, high-yield lines have also shown a gradual decrease in protein concentration when planting dates were later than the second week of May (Beatty et

al., 1982). In the present study, across environments and breeding lines, 10.2 mg g^{-1} decrease in protein concentration was observed between the intermediate and late planting, and an overall lower protein concentration in 2011 (365 mg g^{-1}) than in 2010 (410 mg g^{-1}). It is possible that lower temperatures experienced in the seed-fill period by late-planted soybean may have reduced nodulation (Gardner et al., 2003) and nitrogen fixation, which may have resulted in a reduction in the accumulation of protein and in the differences in protein concentration observed among planting dates.

Oil concentration response to planting date depended on the environment and breeding line. Oil concentration tended to decrease with planting date and, averaged across breeding lines and planting dates, oil concentration was only slightly greater in 2010 (190 mg g^{-1}) than in 2011 (184 mg g^{-1}). Thus, the decrease in protein concentration with planting dates and in 2011 observed in this experiment supports that cool temperatures experienced during seed-fill play a significant role in determining soybean seed composition. However, the exact mechanism responsible for changes in soybean seed composition is not well understood (Wilson, 2004). Oil, on the other hand, showed no clear association with the changes in temperatures observed among environments. Unaccounted differences may be due to other environmental variables affecting the expression of the trait. Nevertheless, early planting would likely enhance seed protein and oil concentrations.

Minimum daily temperatures during the late reproductive period (August and September) have been used to explain differences in fatty acid profiles in previous studies (Hou et al., 2006). Contrarily, Bachlava and Cardinal (2009) reported that daily *average* temperatures correlated well with oleic acid concentration in late-maturing cultivars grown in three locations in North Carolina in two consecutive years. In this study, oleic acid concentration among environments

also coincided with the trend observed in protein concentration and with differences in day *minimum* temperatures. These results agreed with previous studies that reported temperature correlations with seed composition (Kane et al., 1997). Oleic acid concentration tended to decrease in late-planted soybeans, but the effect depended on breeding line. Oliva et al. (2006) showed that high oleic acid lines were unstable when compared to normal oleic lines in 10 environments. Similarly, Carver et al. (1986) observed that oleic acid concentration was greater in warmer environments in a set of lines that had been selected for increased oleic acid concentration for eight cycles of selection. Therefore, the high oleic soybeans may fit better in an early production system.

The trend observed for linolenic acid and saturated fats in this study agreed with previous research. Wilcox and Cavins (1992) and Oliva et al. (2006) reported that both normal and low linolenic acid lines had negative correlations between air temperature during the seed-fill period and linolenic acid concentration. The lower linolenic acid concentration at early planting of some of the lines used in this study may be associated with higher temperature slowing the activity of the enzyme desaturase (adds double bonds to fatty acids). A laboratory study showed that the activity of linoleoyl (18:2) desaturases was dramatically lower in soybean cell cultures at 35°C than the control at lower temperature, which would result in reduced amounts of linolenic acid in the oil. These changes in enzymatic activities have been also proposed as explanation for changes in seed fatty acid composition in other field studies with soybean experiencing different temperatures during the seed development period (Hou et al., 2006). Therefore, for commercial production of low linolenic soybeans, farmers are encouraged to use an early productions system or plant early when using a full-season production system.

Sucrose was the organic component that responded to planting date more consistently. All the breeding lines showed an increase in sucrose concentration with planting date in all environments studied. Similarly, Bellaloui et al. (2011) showed an increase in sucrose and decrease in stachyose at late planting dates for a high-yield soybean line studied under different irrigation regimes. Evidently, delayed planting or double cropping of high-sugar soybeans after barley (*Hordeum vulgare*) or wheat would enhance the sucrose levels for soyfood processing purposes. Seed sugar concentration is probably the organic seed component that has received the least attention in planting dates studies. Growing demand for specialty soybean with modified sugar profile calls for research on management practices that can help maximize this trait.

The greater concentration of seed chemical elements from an early planting, assuming higher temperature during the seed-fill period than for late-planted soybean, agrees with patterns observed in other studies. Gibson and Mullen (2001) reported an increase in concentration of P, K, Ca, and Mg concentrations in the seed when plants were exposed to high temperatures (35°C) in growth chambers during the reproductive seed-fill and maturation period (R5-R8). Changes in seed composition can also be caused by water stress, usually associated with high temperatures in field conditions (Rose, 1988; Piper and Boote, 1999; Wilson, 2004). Samara et al. (2004) studied the effects of drought stress on soybean seed inorganic composition and reported, like Gibson and Mullen (2001), an increase in the concentration of P, K, Ca, molybdenum (Mo) , Mn, Cu, and Zn under water-deficit stress in both greenhouse and field conditions (Rose, 1988; Piper and Boote, 1999). In contrast, Smiciklas et al. (1989) reported a significant lower Ca concentration in drought-stressed soybeans than in a well-watered control. The authors proposed that under water stress conditions chemical elements that are transported in the plant xylem, and therefore, depend on plant transpiration, may reach reproductive organs in low amounts due to

increased stomatal resistance and consequent reduced transpiration that plants exhibit under water stress (Smiciklas et al., 1989). Karlen et al. (1982) reported higher P concentration in plants that were irrigated than plants that were without irrigation. The difference between the treatments may have been caused by an increase in soil P diffusion to the plant roots. The disagreement between these studies suggests that seed inorganic composition may respond differently to the timing, severity, and duration of water-deficit stress (Samarah et al., 2004). The differences that were observed in inorganic seed composition between years in this study need further investigation. Considering that 2011 was cooler in September, but hotter in July and August than 2010, conclusions cannot be drawn on whether the changes in seed inorganic components were due temperature effects on enzymatic reactions involved in chemical element absorption and transport, or due to the effect of water stress on the volume of water that moved through the plant. However, because the plots in this study were fully irrigated, temperature differences rather than water-deficit stress seems to be the most likely explanation. As mentioned above, and unlike the case of organic seed components, this is one of the few studies on the effects of planting date and environment on soybean seed inorganic composition. Nevertheless, the results in the present study indicated that at least until the third week of May, there should not be a detrimental reduction in inorganic seed components due to delaying planting. If market requirements call for a more specific inorganic composition, other planting strategies and management may be needed. Perhaps, breeding for specific inorganic composition would be a more viable and effective option (Graham et al., 1999; Wang et al., 2003; Welch and Graham, 2004). A good example would be breeding small-seeded soybeans with low Ca concentration and soft texture for the natto market.

Delayed harvest effects on organic seed composition

Most of the traits studied did not show significant environment by breeding line by harvest time interaction (Table 6). Therefore, breeding line was not included in the means table and posterior contrast tests. Protein and oil concentrations were not significantly affected by delayed harvest and were the most stable traits (Table 7). Like with planting date, sucrose responded consistently among the four environments. Krober and Collins (1948) reported a significant increase in protein and decrease in sugars when comparing weathered soybeans (delayed harvest) with good quality soybeans. However, the decrease in sucrose was only about 2%. Similarly, Yaklich (1985) observed a small, though not statistically significant, difference in sugar concentration between soybeans harvested at maturity and soybeans harvested six weeks later. In this study, sucrose and stachyose concentration decreased significantly, but only slightly, with delayed harvest (Table 7). The sucrose and stachyose concentrations of R07-2000 (bred for high sucrose/low stachyose) seed were in all environments by harvest time combinations no lower than 78 mg g^{-1} and greater than 3.3 mg g^{-1}, respectively, which were well above/below any commodity soybean and would qualify for seed quality premiums. Harvest delays of the magnitude studied here should not be a major concern for farmers growing high sucrose/low stachyose soybeans.

Differences in oleic acid concentration between harvest dates, although significant, were minimal and would not exclude/prevent these lines from being harvested 14-20 d after maturity. Farmers growing R07-8292 (high oleic line) would be able to obtain benefits from selling high oil soybeans and from having flexibility to harvest the crop later than maturity if other farm activities have, at that time, higher priority (harvest a different crop at the farm).

Saturated fatty acids increased slightly with delayed harvest in three of the four environments studied (Table 7). Although significant, these differences in fatty acid composition would not

justify any change in harvest practices in order to attain low linolenic or low sats soybeans. There have not been field experiments conducted investigating the effect of delayed harvest on soybean seed fatty acids. Most research focused on the effects of accelerated aging test or different storage conditions (Locher and Bucheli, 1998). These studies have been valuable because they have shown how seed components change under adverse and extreme conditions, which may be somehow extrapolated to a delayed harvest field experiments. Linolenic acid increased significantly with time in soybeans seed stored at different temperatures (20, 30, 40°C)(Trawatha et al., 1995). In contrast, Priestley and Leopold (1983) showed no change in fatty acid composition in aged seed. The mechanism that was proposed to explain changes in seed fatty acid composition in aged seed was lipid peroxidation. It appears that changes in fatty acid composition caused by weathering, storage, and natural aging of the seed are still unclear (Stewart and Bewley, 1980). Under the conditions in the present study, there should be no concerns for possible/significant changes in fatty acids due to delayed harvest. Perhaps, longer delays may have a significant impact on fatty acid composition.

Delayed harvest effects on inorganic seed composition

All chemical elements concentrations were lower in the delayed harvest seed than in seed harvested at maturity (Table 9), and except for Ca, Fe and Cu, showed a significant environment by harvest time interaction (Table 8). The lack of interaction suggests that the differences in Ca, Fe, and Cu concentration between seed harvested at maturity and seed harvested 14-20 d later did not differ across environments (Table 9). At maturity, chemical element uptake and remobilization among plant parts is reduced to negligible amounts or fully stopped due to the absence of phloem loading. Further, unlike organic components, seed chemical elements do not undergo catabolic processes and are not lost in seed respiration. The differences that were

observed still left chemical element concentrations well within the expected range for healthy soybean seeds (Raboy et al., 1984; Rengel et al., 1999; Moraghan et al., 2006) but, based on previous literature, no explanation for those small changes could be provided.

In conclusion, of the two management practices studied here, planting date had a larger impact on seed composition. Results for planting dates agreed with previous studies and showed that lines developed for Arkansas followed a similar response to that observed in other latitudes. Late planting decreased protein, oleic acid, did not have a large effect on saturated fatty acids, and increased sucrose. Patterns of seed composition response to planting date supported that temperature during the seed-fill period governed organic seed composition. Unless producers grow specialty cultivars bred for high sucrose or low Ca, they should plant soybeans no later than the third week of May in Arkansas in order to prevent the risk of experiencing yield losses or significant changes in seed composition. It may be possible that tofu or edamame producers would receive a premium for an increase in seed sucrose concentration, possibly justifying purposeful delay in planting or choosing a double-cropped system (soybean after wheat/barley).

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Table 1. Seed traits of soybean lines used in the planting date and delayed harvest study.

Genotype‡	Trait
R05-1772	high protein†
R05-1415	high protein†
R05-655	high oil
R02-6268F	high oil
R07-8292	high-oleic acid
R05-5346	low linolenic acid
R06-814	low saturated fatty acids (stearic + palmitic acid)
R07-2000	high sucrose, low stachyose, high inorganic phosphorus
AG5605	high yield

‡ see Chen et al. (2008) and Chen et al. (2011) for more details on these lines.

† all lines, except AG5605 which is property of Asgrow Seed Company, were developed at the University of Arkansas soybean breeding program.

Table 2. Analysis of variance for organic seed components of soybean breeding lines with modified seed composition grown at two Arkansas locations (Kibler and Fayetteville) during two years (2010, 2011).

Source of effects	Dfn†	Df Den‡	Yield§	Pro	Oil	Ole	Lin	Sats	Suc	Sta	Pi
Environment (Env)	3	8	*	***	*	*	***	**	***	***	***
Planting date (Pd)	2	16	NS	***	NS	NS	*	*	***	*	**
Env x Pd	6	16	NS	NS	NS	*	***	*	NS	*	*
Breeding line (Bl)	8	183	***	***	***	***	***	***	***	***	***
Env x Bl	24	183	***	***	***	***	***	**	***	***	**
Pd x Bl	16	183	NS	NS	*	***	*	*	*	NS	***
Env x Pd x Bl	48	183	NS	*	*	**	*	NS	NS	NS	NS

† degrees of freedom of numerator in F test.

‡ degrees of freedom of denominator in F test.

§ Yield in kg ha^{-1}, Pro = protein concentration (mg g^{-1}); Oil concentration in mg g^{-1}; Ole = oleic acid concentration (mg g^{-1}); Lin = linolenic acid concentration (mg g^{-1}); Sats = saturated fatty acids (palmitic + stearic acids) (mg g^{-1}); Suc = sucrose concentration (mg g^{-1}); Sta = stachyose concentration (mg g^{-1}); Pi = inorganic phosphorus concentration (µg g^{-1}).

NS not significant at 0.05 probability level.

* significant at the 0.05 probability level.

** significant at the 0.01 probability level.

*** significant at the 0.001 probability level.

Table 3. Yield and seed organic composition of soybean breeding lines with modified seed composition as influenced by planting date (early, intermediate, late) averaged across environments.

Breeding line	Yield†	Pro	Oil	Ole	Lin	Sats	Suc	Sta	Pi
AG5605									
early	3255	373	195	245	60	136	40	42	223
int‡	3107	379	193	230	59	138	44	42	214
late	3101	364	200	237	63	139	56	44	230
R02-6268F									
early	2704	375	201	240	65	138	51	40	216
int	3007	373	197	249	60	142	54	39	220
late	2659	367	196	261	65	139	64	41	237
R05-1415									
early	2621	418	175	178	76	147	43	39	184
int	2863	417	177	190	77	139	43	43	169
late	2555	409	177	199	72	149	53	39	234
R05-1772									
early	2527	414	178	194	72	145	43	38	225
int	2476	412	179	201	69	143	47	40	205
late	2391	402	180	217	70	144	53	41	242
R05-5346									
early	2588	379	194	262	36	135	48	40	228
int	2959	375	197	263	34	136	55	41	195
late	2710	365	196	273	38	139	65	43	226
R05-655									
early	2452	372	201	244	59	137	44	42	226
int	2627	370	204	237	58	138	50	43	191
late	2938	361	204	244	63	138	61	44	211
R05-814									
early	1825	411	177	256	45	78	37	42	247
int	2337	404	180	241	43	75	43	42	232
late	1899	397	178	253	48	84	50	44	231
R07-2000									
early	2746	394	187	253	66	130	90	1	1778
int	2859	395	185	258	64	130	92	1	1762

late	2649	383	182	256	74	130	109	1	1994
R07-8292									
early	2746	41	41	41	41	41	41	41	41
int	2859	43	43	43	43	43	43	43	43
late	2649	39	39	39	39	39	39	39	39
LSD§	336	4	3	12	3	5	3	2	40

† yield in kg ha^{-1}, Pro = protein concentration (mg g^{-1}); Oil concentration in mg g^{-1}; Ole = oleic acid concentration (mg g^{-1}); Lin = linolenic acid concentration (mg g^{-1}); Sats = saturated fatty acids (palmitic + stearic acids) (mg g^{-1}); Suc = sucrose concentration (mg g^{-1}); Sta = stachyose concentration (mg g^{-1}); Pi = inorganic phosphorus concentration (µg g^{-1}).

§ least significant difference at 0.05 probability level for least square means breeding line by planting date combination within the same trait.

‡ intermediate planting date.

Table 4. Analysis of variance for inorganic seed components of breeding lines with modified seed composition grown at two Arkansas locations (Kibler and Fayetteville) during two years (2010, 2011).

Source of effects†	Dfn‡	Dfd §	P¶	K	Ca	Mg	S	Fe	Mn	Zn	Cu
Environment (Env)	3	8	**	***	***	***	***	***	***	***	**
Planting date (Pd)	2	16	*	*	***	***	**	NS	***	**	NS
Env x Pd	6	16	**	*	*	**	***	*	**	***	*

† because they had not been bred for any specific inorganic seed composition, breeding lines were considered a random effect in the analysis of inorganic composition data, and therefore, were included in the error term by the PROC MIXED procedure in SAS.

‡ degrees of freedom of numerator in F test.

§ degrees of freedom of denominator in F test.

¶ P, K, Ca, Mg, and S in mg g^{-1}; Fe, Mn, Zn and Cu in mg kg^{-1}.

NS not significant at 0.05 probability level.

* significant at the 0.05 probability level.

** significant at the 0.01 probability level.

*** significant at the 0.001 probability level.

Table 5. Seed chemical element concentrations of soybean breeding lines with modified seed composition as influenced by planting date (early, intermediate, late) and averaged across breeding lines and environment.

	P†	K	Ca	Mg	S	Fe	Mn	Zn	Cu
early	7.0	21.2	3.3	2.6	3.7	64	39	43	17
int‡	7.5	21.7	3.3	2.8	3.8	62	38	45	16
late	7.1	20.8	2.9	2.6	3.7	62	35	46	16
LSD§	0.5	0.7	0.3	0.1	0.2	3	2	2	1

† P, K, Ca, Mg, and S in mg g^{-1}; Fe, Mn, Zn and Cu in mg kg^{-1}.

‡ intermediate planting date.

§ least significant difference at 0.05 probability level for least square means of planting date.

Table 6. Analysis of variance for organic seed components of breeding lines with modified seed composition grown at two Arkansas locations (Kibler and Fayetteville) with optimum planting date for the full-season crop during two years (2010, 2011).

Source effects	Dfn†	Dfd‡	Pro§	Oil	Ole	Lin	Sats	Suc	Sta	Pi
Environment (Env)	3	8	***	NS	NS	**	***	***	*	*
Breeding line (Bl)	8	57	***	***	***	***	***	***	***	***
Env x Bl	24	57	***	***	***	**	*	*	NS	NS
Harvest time (Ht)	1	65	NS	NS	NS	*	***	***	*	***
Env x Ht	3	65	NS	NS	**	***	***	***	***	***
Bl x Ht	8	65	NS	NS	NS	NS	NS	*	NS	NS
Env x Bl x Ht	24	65	NS	NS	NS	NS	NS	NS	NS	**

† degrees of freedom of numerator in F test.

‡ degrees of freedom of denominator in F test.

§ pro = Protein concentration (mg g^{-1}); Oil concentration in mg g^{-1}; Ole = oleic acid concentration (mg g^{-1}); Lin = linolenic acid concentration (mg g^{-1}); Sats = saturated fatty acids (palmitic + stearic acids) (mg g^{-1}); Suc = sucrose concentration (mg g^{-1}); Sta = stachyose concentration (mg g^{-1}); Pi = inorganic phosphorus concentration (µg g^{-1}).

NS not significant at 0.05 probability level.

* significant at the 0.05 probability level.

** significant at the 0.01 probability level.

*** significant at the 0.001 probability level.

Table 7. Seed organic composition (averaged across breeding lines) as influenced by delayed harvest grown at two Arkansas locations (Kibler and Fayetteville) with optimum planting date for the full-season crop during two years (2010, 2011).

		Environment§					
Trait†	**Harvest time‡**	**2010KIB**	**2010FAY**	**2011KIB**	**2011FAY**	**Mean¶**	**LSD#**
Pro	**Mat**	414	417	369	362	391	5
	Dh	409	419	368	364	390	
Oil	**Mat**	191	189	185	183	187	4
	Dh	190	186	185	185	186	
Ole	**Mat**	265	253	235	255	252	9
	Dh	253	257	256	254	255	
Lin	**Mat**	59	62	61	42	56	3
	Dh	61	60	41	52	53	
Sats	**Mat**	124	123	137	138	130	2
	Dh	126	127	152	134	135	
Suc	**Mat**	47	59	49	61	54	2
	Dh	45	51	47	58	50	
Sta	**Mat**	31	35	38	42	36	2
	Dh	38	36	36	39	37	
Pi	**Mat**	430	375	344	374	381	31
	Dh	465	439	385	359	412	

† Pro = protein concentration (mg g^{-1}); Oil in mg g^{-1}; Ole = oleic acid concentration (mg g^{-1}); Lin = linolenic acid concentration (mg g^{-1}); Sats = saturated fatty acids (palmitic + stearic acids) (mg g^{-1}); Suc = sucrose concentration (mg g^{-1}); Sta = stachyose concentration (mg g^{-1}); Pi = inorganic phosphorus concentration (µg g^{-1}).

‡ Mat = seed harvested at maturity; Dh = seed harvested 14-20 d after maturity.

§ 2010KIB = data from 2010 Kibler, 2010FAY = data from 2010 Fayetteville, 2011KIB = data from 2011 Kibler, 2011FAY = data from 2011 Fayetteville.

¶ trait mean for the harvest time date across environments.

least significant difference at 0.05 probability level for least square means for harvest time by environment combinations within the same trait.

Table 8. Analysis of variance for inorganic seed components of soybean breeding lines with modified seed composition grown at two Arkansas locations (Kibler and Fayetteville) with optimum planting date for the full-season crop during two years (2010, 2011).

Source of effects	Dfn†	Dfd‡	P§	K	Ca	Mg	S	Fe	Mn	Zn	Cu
Environment (Env)	3	8	**	***	***	***	***	***	***	***	*
Harvest time (Ht)	1	8	***	***	***	***	***	***	***	**	*
Env x Ht	3	8	*	***	NS	**	*	NS	*	*	NS

NS not significant at 0.05 probability level.

*significant at the 0.05 probability level.

**significant at the 0.01 probability level.

***significant at the 0.001 probability level.

† degrees of freedom of numerator in F test.

‡ degrees of freedom of denominator in F test.

§ P, K, Ca, Mg, and S in mg g^{-1}; Fe, Mn, Zn and Cu in mg kg^{-1}.

Table 9. Seed chemical element concentrations for soybean breeding lines with modified seed composition as influenced by delayed harvest and evaluated at two Arkansas locations (Kibler and Fayetteville) with optimum planting date for the full-season crop during two years (2010, 2011).

		Environment§					
Trait†	**Harvest time‡**	**2010KIB**	**2010FAY**	**2011KIB**	**2011FAY**	**Mean¶**	**LSD#**
P	**Mat**	7.9	8.0	7.0	6.7	7.4	0.5
	Dh	7.2	6.9	6.5	6.4	6.7	
K	**Mat**	23.9	23.7	20.7	19.2	21.9	0.8
	Dh	20.3	19.7	19.4	18.1	19.4	
Ca	**Mat**	4.1	3.7	3.0	2.2	3.3	0.3
	Dh	3.8	3.3	2.8	2.1	3.0	
Mg	**Mat**	3.0	2.9	2.7	2.4	2.8	0.1
	Dh	2.7	2.5	2.5	2.3	2.5	
S	**Mat**	3.9	4.3	3.4	3.6	3.8	0.2
	Dh	3.6	3.8	3.2	3.5	3.5	
Fe	**Mat**	68	68	56	55	62	4
	Dh	58	61	51	51	55	
Mn	**Mat**	35	57	29	32	38	4
	Dh	32	48	26	31	34	
Zn	**Mat**	49	50	38	46	46	3
	Dh	44	44	37	45	43	
Cu	**Mat**	16	17	16	15	16	1
	Dh	15	16	15	15	15	

† P, K, Ca, Mg, and S in mg g^{-1}; Fe, Mn, Zn and Cu in mg kg^{-1}.

‡ Mat = seed harvested at maturity; Dh = seed harvested 14-20 d after maturity.

§ 2010KIB = data from 2010 Kibler, 2010FAY = data from 2010 Fayetteville, 2011KIB = data from 2011 Kibler, 2011FAY = data from 2011 Fayetteville.

¶ trait mean for the harvest time date across environments.

least significant difference at 0.05 probability level for least square means for harvest time by environment combinations within the same trait.

Table 10. Monthly average daily maximum temperature (T max in °C), daily minimum temperature (T min in °C), and total monthly rainfall (in mm) for the growing season and environments in which breeding lines with modified seed composition were evaluated in this study.

		2010		2011	
		Kibler	**Fayetteville**	**Kibler**	**Fayetteville**
May	T max	27.7	24.9	25.8	22.2
	T min	16.3	15.0	14.6	13.0
	Rainfall	111	209	214	226
June	T max	33.9	31.5	34.9	31.7
	T min	22.5	20.8	22.2	21.6
	Rainfall	121	42	20	27
July	T max	33.7	31.5	38.5	36.3
	T min	23.4	22.4	24.4	23.8
	Rainfall	0	353	14	19
August	T max	36.5	34.7	36.1	34.9
	T min	23.1	22.5	22.9	22.6
	Rainfall	101	0	105	98
September	T max	29.4	28.5	28.9	25.8
	T min	18.0	16.6	13.2	12.6
	Rainfall	161	216	38	142
October	T max	25.1	22.1	24.6	22.1
	T min	8.5	8.5	9.5	8.8
	Rainfall	36	42	121	73

Figure 1. Planting date [early, intermediate (int), late] effects on soybean seed composition of soybean breeding lines grown at two Arkansas locations (Kibler and Fayetteville) during two years (2010, 2011).

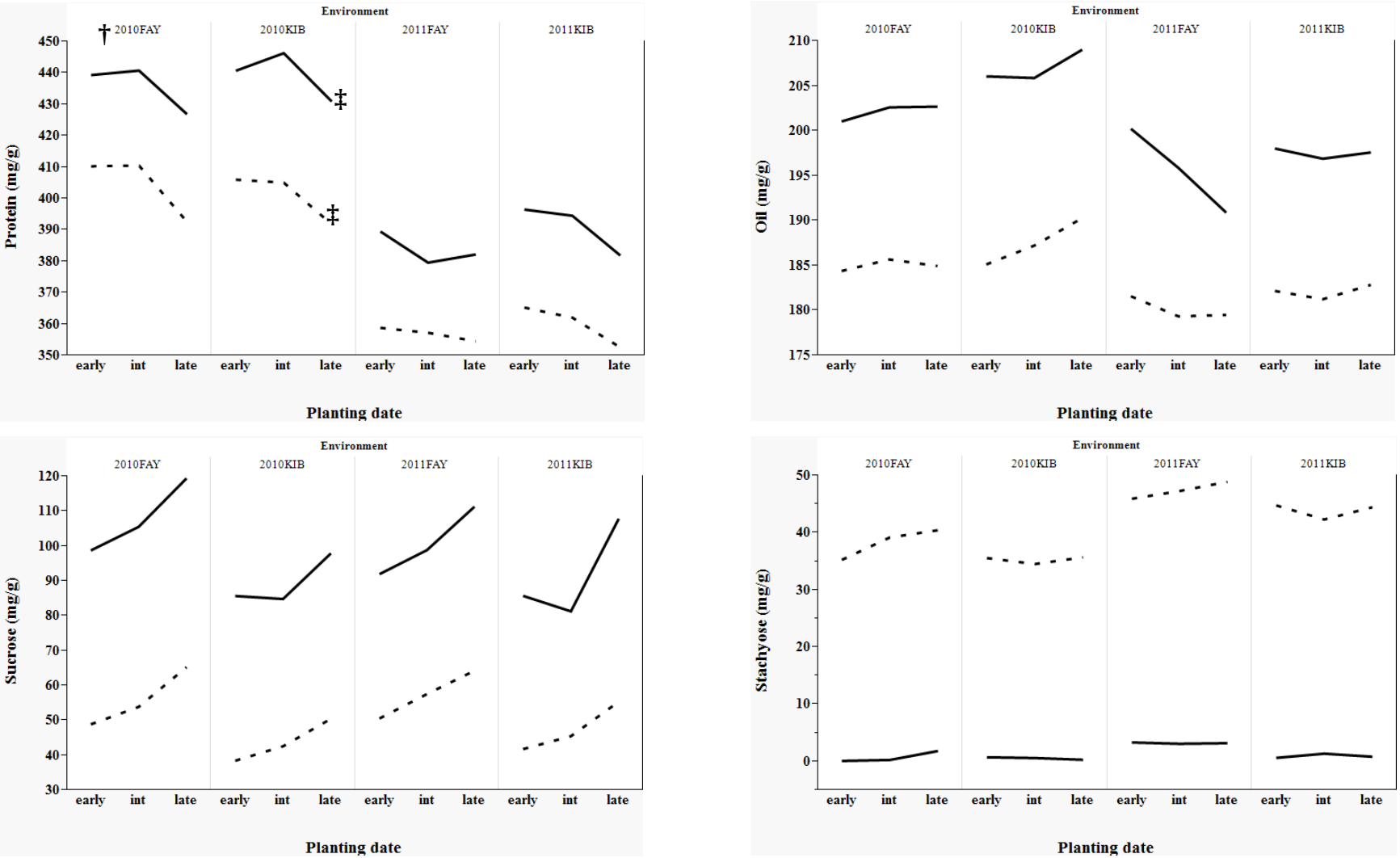
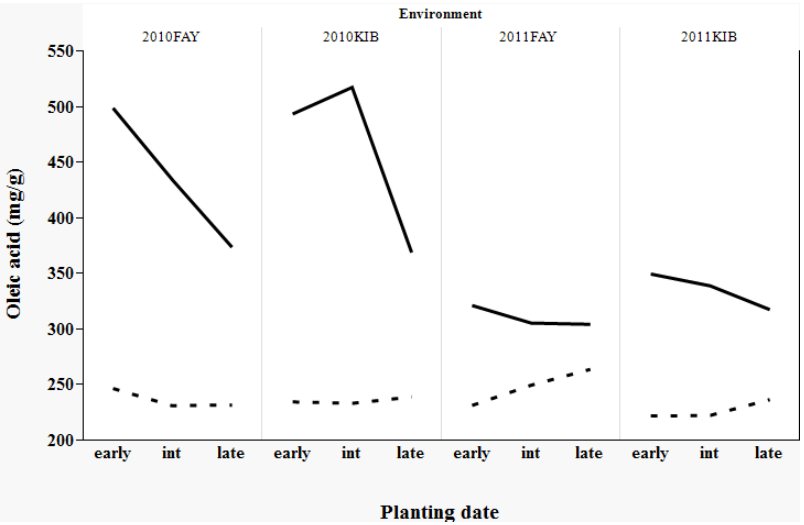
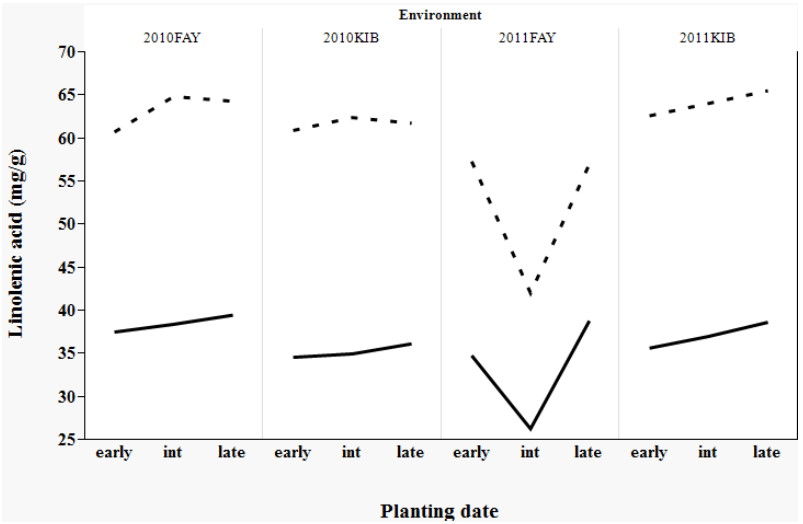
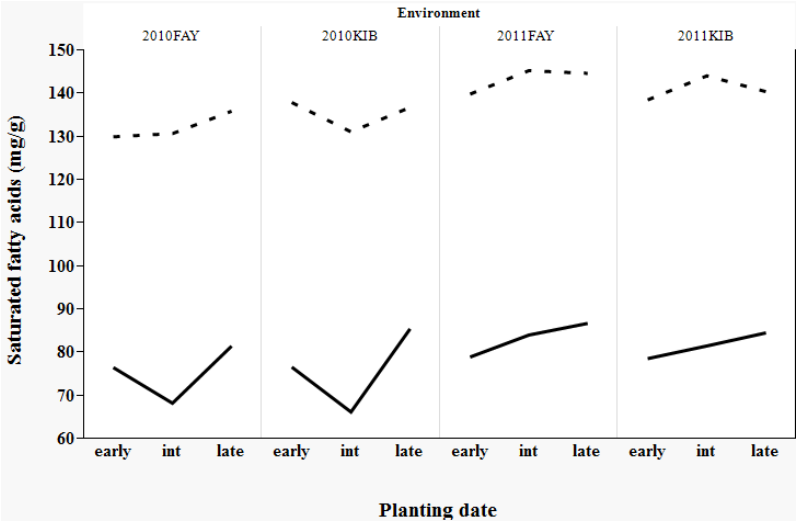


Figure 1 continued.







† 2010KIB = data from 2010 Kibler, 2010FAY = data from 2010 Fayetteville, 2011KIB = data from 2011 Kibler, 2011FAY = data from 2011 Fayetteville.

‡ The solid line represents the breeding line or the average of breeding lines that was/were bred for the seed component presented in the graph. The dashed line represents the average of lines that were not bred for the trait presented in the graph. For example, for protein concentration, the solid line is the average of breeding lines R05-1415 and R05-1772, whereas the dashed lines is the average of all other lines. See Table 1 for the other seed components.

V. Association between soil and leaf chemical elements, and soybean seed composition

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Abbreviations: EC, electrical conductivity; HPLC, high performance liquid chromatography; Kib, Kibler, AR; Fay, Fayetteville, AR; Pro, protein; Ole, oleic acid; Lin, linolenic acid; Sats, saturated fatty acids; Suc, sucrose; Sta, stachyose; Pi, inorganic phosphorus; P, phosphorus; K, potassium; Ca, calcium; Mg, magnesium; S, sulfur; Fe, iron; Mn, manganese; Zn, zinc; Cu, copper; B, boron; dfn, degrees of freedom of numerator; dfd, degrees of freedom of denominator, LSD, least significant difference.

ABSTRACT

Environmental variables may play a major role in determining the seed composition of soybean. Maximizing the expression of seed quality traits by understanding how they are affected by environmental variables will help develop high quality nutritious soybeans. In this study, the potential relationship between soil properties and leaf chemical element concentration, with seed composition was studied. Eight specialty soybean breeding lines were grown at two Arkansas locations with three replications in 2011. Before the reproductive period soil and leaf samples were taken from each plot; soil samples were analyzed for particle size distribution, electrical conductivity, pH, and ten chemical elements: phosphorus, potassium, calcium, magnesium, sulfur, iron, manganese, zinc, copper and boron, while leaf samples were analyzed for the chemical elements. At maturity, seed samples were analyzed for the same ten chemical elements as leaf and soil samples, plus protein, oil, fatty acids, and sugars. All leaf chemical elements were within the adequate levels for crop production at both locations. Overall, seed composition of breeding lines did not differ between locations and no significant changes of breeding line ranking between locations were observed. Attempting to modify seed composition by nutrient fertilization may not be profitable, as no association was observed between leaf or soil chemical elements, and seed composition. These findings may provide a starting point for future studies on fertilization and management practices that improve soybean seed quality.

INTRODUCTION

Understanding the contribution of environmental variables to seed composition may help develop cultural and breeding strategies to develop and produce high quality soybeans, especially due to the increasing global need of more nutritious food. Plant nutrient uptake is active during vegetative plant growth as chemical elements are incorporated into proteins to activate enzymes and contribute to the charge balance in the cell (Gardner et al., 2003). During the reproductive period, chemical elements can be remobilized to seeds from vegetative tissue via the phloem, and to a lesser extent, newly uptaken by the roots via the xylem, transferred to phloem and transported to the reproductive organs (Marschner, 1997; Grusak et al., 1999). Decreases in leaf chemical elements concentration during the reproductive period has been well documented, and has been used as evidence for nutrient remobilization in several crops (Hocking and Pate, 1977; Loneragan et al., 1980; Pearson and Rengel, 1994; Waters and Sankaran, 2011). Thus, factors affecting the availability of nutrients in the soil and the rate of uptake and remobilization of chemical elements by the plant have the potential to affect seed inorganic composition. Further, because chemical elements are essential for plant metabolic processes, those factors also have the potential to significantly affect the organic composition of the seed (Wilson et al., 1982).

Kravchenko and Bullok (2000) studied the influence of field elevation, slope and curvature on soybean seed protein and oil concentration in several fields in Illinois from 1994 and 1997. The three variables were positively correlated with protein concentration in most of the fields studied. Oil was less affected by topography than protein concentration. The effects of topography interacted with weather patterns, which played an important role in crop growth and seed composition. Bellaloui et al. (2009b) measured soil chemical element concentration and

soybean seed composition of field sections that differed in yield potential. Areas of low yield potential exhibited lower protein and oleic acid concentration than areas of medium and high yield potential. Areas of low yield potential had lower soil organic matter, and extractable sodium (Na), calcium (Ca), potassium (K), boron (B), and zinc (Zn) concentrations than areas of medium and high yield potential. Differences in protein concentration between areas were hypothesized to be caused by differences in soil sodium (Na), B, and Zn; which are important in enzymatic reactions involved in protein and nitrogen metabolism. According to Bellaloui et al. (2009b) differences in soil K between the areas studied were proposed to have determined differences in photosynthetic capacity and consequent alteration of seed protein and oil concentrations (Bellaloui et al., 2009b). Similarly, Poutaraud and Girardin (2004) studied the correlation between soil chemical properties and the seed alkaloid concentration in meadow saffron (*Crocus sativus*) seeds in France. A significant positive linear relationship was observed between Ca and Ca + cobalt (Co) concentration in the soil and seed alkaloid concentration. The fact that the enzymes in the alkaloid synthesis pathways needed Co and that Ca prevents alkaloids from entering cell compartments, where otherwise they would be degraded, was proposed as the explanation for the results observed. No such soil-leaf-seed chemical composition correlation studies have been conducted for Arkansas grown soybeans. It is hypothesized that soil variables may have a significant effect on seed composition, as they greatly contribute to plant nutrient availability their mobilization and overall plant growth.

The objectives of this study were to evaluate possible associations between soil properties and chemical element concentrations in the leaf with seed composition in four types of specialty soybeans: high protein, high oil, modified fatty acids, low phytate/low stachyose grown at two Arkansas locations in 2011.

MATERIALS AND METHODS

Field experiment

This experiment was part of a larger experiment described in Jaureguy et al. (2012). Eight breeding lines with modified seed composition and a high-yield check were used for this study (Table 1). Breeding lines were arranged in a randomized complete block design with three blocks. Each plot consisted of a 5-m, four-row plot. Seeding rate was 30 seeds per meter. The experiment was planted at the Arkansas Agricultural Research and Extension Center in Fayetteville, AR, and at the University of Arkansas Vegetable Research Station in Kibler, AR, in 2011. The soil at the Fayetteville location is mapped as Captina silt loam (fine-silty, siliceous, active, mesic Typic Fragiudult) and described as very deep, moderately well-drained soil developed on a thin mantle of silty material. The previous crop in the Fayetteville field was wheat (*Triticum aestivum*). Kibler soil is mapped as Roxana silt loam (Soil Survey Staff, 2011) and described as well-drained, moderately permeable soil formed in a stratified loamy alluvium (Garner and Cox, 1979). The previous crop in the Fayetteville field was soybean. Row spacing was 100 cm and 90 cm and planting dates were June 2nd and May 31st for Fayetteville (planted on beds) and Kibler (planted on flat ground), respectively. Field plots were fully irrigated (overhead at Kibler; furrow irrigation at Fayetteville) and managed during the growing season using standard cultural practices adopted for full-season soybean production in Arkansas (Tacker and Vories, 1998). Cultural practices included tillage with chisel plow and disc, and fertilization based on soil test results and recommendation of University of Arkansas Cooperative Extension Service. Weed control was performed by applying pre-plant and post-emergence herbicides (e.g. glyphosate), at label rates. At maturity, the two center rows of the plots were harvested using a plot combine. Seed was stored for phenotypic analysis.

Soil sampling, soil chemical element composition and soil texture analysis

Soil samples were collected from top 12.5 cm of each plot about one week before the beginning of the reproductive period (flower initiation). Twelve soil cores (three per row) were extracted with a push probe with 2.5 cm diameter, mixed in a plastic bucket and stored in a box. Soil samples were dried in a temperature controlled shed (drier) at the Arkansas Agricultural Research and Extension Center in Fayetteville, AR, ground with an industrial bench grinder, sieved through a screen and submitted to the University of Arkansas Soil Testing and Research Laboratory in Fayetteville, AR. Chemical elements were extracted with Mehlich-3 extractant solution in a 1:10 soil/extractant solution ratio. Samples were analyzed for elements [phosphorus (P), K, Ca, magnesium (Mg), sulfur (S), iron (Fe), manganese (Mn), Zn, copper (Cu), and B] concentration using a Spectro Arcos Inducted Coupled Plasma Mass Spectrometer (Spectro Analytical Instruments, Mahwah, NJ). Soil pH and electric conductivity were determined with an electrode on a 1:2 soil/water solution. Data were expressed in milligram of element per kilogram of dry soil (mg kg^{-1}).

For particle size analysis, a 50 g sample of oven-dried sieved soil was weighed on a flattop scale, mixed with 50 ml of 100 g L^{-1} sodium hexametaphosphate solution in a 250 ml Erlenmeyer flask, and brought to a total volume of 1 L with tap water in a 1 L sedimentation cylinder. The solution was allowed to sit on the laboratory bench for 24 h. The next day a blank hydrometer reading (R_0) in a sedimentation cylinder with only water was taken. Afterwards, each sample solution was vigorously mixed with a metal plunger, and 40 s after the last stroke, the hydrometer was lowered into the solution to take a reading ($R_{40\,s}$). The reading, including mixing, was repeated two more times for a total of three replications. A final hydrometer reading was taken two hours later ($R_{2\,hs}$). The blank hydrometer reading was corrected for temperature

deviations by adding 0.4 for each degree C above 20°C and subtracting 0.4 for each degree C below 20°C. The following formulas (Gee and Or, 2002) were used to estimate the percentage of clay, silt and sand in the sample:

$$\% \text{ silt + clay} = \frac{(R_{40\ s} - R_{0,\ corrected})}{\text{oven-dry soil weight in g}} \times 100$$

$$\% \text{ clay} = \frac{(R_{2\ hs} - R_{0,\ corrected})}{\text{oven-dry soil weight in g}} \times 100$$

Sand % was determined by the formula % sand = 100 - (% silt - % clay), silt % was determined by the formula % silt = 100% - (% clay - % sand).

Leaf composition analysis

Leaf chemical element analysis. Composite samples of the uppermost fully developed trifoliates were collected randomly from the four rows of each plot the same day as when soil samples were collected. Leaf samples were dried in a temperature controlled shed (drier) at the Arkansas Agricultural Research and Extension Center in Fayetteville, AR for 2 d, ground with a bench grinder to a fine powder, sieved through a mesh screen with 0.84 mm opening, and stored in coin envelopes. Samples were digested using concentrated HNO_3 and 30% hydrogen peroxide on a heating block system (Environmental Express, Mt. Pleasant, SC). Samples were analyzed using Spectro Arcos Inducted Coupled Plasma Mass Spectrometer (Spectro Analytical Instruments, Mahwah, New Jersey). Data were presented as percentage of dry tissue weight (%) for P, K, Ca, Mg, S, and as milligram per kilogram of dry tissue weight (mg kg^{-1}) for Fe, Mn, Zn and Cu.

Seed composition analysis

Protein and oil analysis. Samples containing 20-25 g of seed were sent to the USDA research facility at Peoria, IL or to the University of Missouri Delta Center at Portageville, MO for protein, oil and moisture analysis in a FOSS® (Eden Prairie, MN) near infrared transmittance instrument. Light of specific wave length interacts with the sample's organic compounds (e.g. protein and oil), which then transmit different light intensity depending on their concentration in the sample. Protein and oil concentration were presented in milligrams per gram of seed (mg g^{-1}) and in dry-weight basis.

Fatty acid analysis. Samples containing five seeds were sent the DNA facility at Iowa State University in Ames, IA. Fatty acid concentrations [oleic acid, linolenic acid, and saturated fatty acids (sats)] were determined by gas chromatography according to the methods developed by Hammond (1991)**.** The fatty acid concentration was presented in milligrams per total grams of oil (mg g^{-1}) and on an "as is" basis.

Sugar analysis. Each seed sample was processed separately for sugar extraction, sugar fractionation and identification, and sugar quantification by high-performance liquid chromatography (HPLC). For a detailed description on the HPLC methods see Jaureguy et al. (2012). Sugar data were presented in milligrams of sugar per gram of seed (mg g^{-1}) and on an "as is" basis.

Inorganic phosphorus (Pi) analysis. An inverse relationship between phytate and inorganic phosphorus has been reported for lines derived from V99-5089 and other low phytate germplasm sources (Maupin et al., 2011). A sample of 0.1 g of soybean seed fine powder was weighed in a 2.0 ml centrifuge tube. One ml of extraction buffer (deionized water, 12.5 % trichloroacetic acid, 1 M $MgCl_2$) was added, and the solution was vortexed until homogeneous. The solution was incubated overnight (≈16 h) at 4ºC. Then, the sample was vortexed, incubated at room

temperature for 30 min, and centrifuged at 1400 *g* for 4 min. Samples were stored at 4ºC until Pi analysis. A 10 µl sample of each extract was placed in a well of a flat bottom 96-well plate (Becton Dickinson, Franklin Lakes , NJ), containing 90 µl of deionized water. The plates included Pi standards consisting of proportional dilutions of K_2PO_4 in deionized water. Standards and samples were allowed to react with 100 µl of Chen's reagent (6 N sulfuric acid, 2.5% amomium molybdate, 10% abscorbic acid, deionized water) for 1 h, and absorbance at 882 nm was measured in a plate reader (Biotek, Winooski, VT). Samples were run in batches of 96 samples, and all the samples were run with the same batch of reagents. A sample of CX1834-1-6 (high Pi/low phytate) and Osage (low Pi/normal phytate) were also included in each batch of samples as controls for the extraction process. Calculation of Pi concentration in samples was conducted by linear extrapolation using the formula µg Pi = area/curve slope. The conversion factor was calculated according to the changes in concentration that Pi undergoes during extraction procedures. Data were presented as micrograms of element per gram of seed ($\mu g\ g^{-1}$) and on an "as is" basis.

Chemical element analysis. Seed samples were ground in a coffee grinder (Krups®, Shelton, CT) and the powder was screened through a 450 µm sieve (VWR International, West Chester, PA). Samples were stored in air-tight 7.5 x 10 cm plastic bags (Uline, Dallas, TX) and submitted to the University of Arkansas Soil Testing and Research Laboratory in Fayetteville, AR. Samples were digested using concentrated HNO_3 and 30% hydrogen peroxide on a heating block system (Environmental Express, Mt. Pleasant, SC). Samples were analyzed using Spectro Arcos Inducted Coupled Plasma Mass Spectrometer (Spectro Analytical Instruments, Mahwah, New Jersey). Data were presented as mg of chemical element per gram of seed ($mg\ g^{-1}$) for P, K, Ca,

Mg, S, and as milligram of element per kilogram of seed (mg kg^{-1}) for Fe, Mn, Zn and Cu, and on an "as is" basis.

Statistical analysis

Analysis of variance on soil, leaf and seed composition data were performed considering location and breeding line as fixed factors, and block as random factor using the PROC MIXED procedure in SAS (SAS Institute, 2002). Significant interactions were further investigated with the "slice" option, which allowed testing main effects or interactions at different levels of other main effects. Least-square means were compared using the "contrast" option.

Pearson's correlation coefficients between measured variables were estimated using the CORR procedure in SAS (SAS Institute, 2002) and using measurements from each replication, and for each location separately. In addition, the sum of squares for each of the factors of the model: soil element concentration + leaf element concentration + error = seed element concentration was calculated as a measure of the contribution of soil element concentration and leaf element concentration to the variability observed in the seed concentration. This analysis was performed for each location separately.

RESULTS AND DISCUSSION

Soil characteristics

The soil particle size analysis showed that both soils used in this study were, as previously reported (Garner and Cox, 1979; Soil Survey Staff, 2011), silt loams. However, the clay content was significantly greater at the Kibler site, whereas the sand content was significantly greater at the Fayetteville site (Table 2). Silt content did not differ between locations. Soil of the Fayetteville site exhibited lower pH than Kibler and was slightly acidic. Although soil pH is known to affect plant nutrient availability, it did not prevent plants grown in Fayetteville from

incorporating enough nutrients to reach adequate levels in the leaf (see below). Soil electrical conductivity differed between locations, but both were below the range (>500 μmhos/cm) within which plant injury due to high soil salt concentration may be expected. Soil at the Kibler site had greater concentrations of all soil chemical elements analyzed except for P and Fe, which were not different between locations, and S, which was greater in Fayetteville (Table 2). Soil P concentrations were optimum at both locations, but soil K, Zn and Cu, and S concentrations were below optimum in Fayetteville and Kibler, respectively. Calcium, Mg, Mn, and B concentrations were above the optimum level at both locations. In contrast, Kibler soil exhibited more optimum chemical properties and it was, overall, a better soil for soybean growth (Table 2). The differences in soil chemistry among locations are possibly due to natural differences between the soil and the effect of previous management practices (e.g. tillage, and fertilization) at the two locations.

Leaf chemical element composition

None of the chemical elements showed significant location by breeding line interaction, which suggested that the leaf chemical element concentration of all lines responded similarly to the environment, and without significant changes in the ranking of the breeding lines between locations (Table 3). Thus, whether breeding lines had been selected for specific seed components did not influence the ability of the plants to take up inorganic nutrients from the soil and supply them to the metabolic systems during growth and development. However, leaf Mg, Fe, Mn, Zn, and B concentrations were, averaged across breeding lines, significantly different among locations. Breeding lines had greater concentrations of leaf Mg, Fe, Zn, and B at Kibler, but greater concentration of leaf Mn at Fayetteville (Table 4). All lines at both locations had adequate leaf chemical element concentrations for normal growth (Table 4) that would not result

in a recommendation for fertilization. The reason and consequences of plants having significantly higher/lower concentrations in one location than the other should be studied in the future; particularly in the context of other variables that are different between locations and that can affect the ability of the plant to uptake certain inorganic nutrients.

Seed organic and inorganic composition

Oleic and linolenic acids showed significant location by breeding line interaction, which suggested that the expression of these two traits depended on both breeding line and location (Table 5). Four breeding lines (R05-1415, R05-1772, R05-655, R07-2000, R07-8292) showed greater oleic acid concentration in Fayetteville, but only R07-8292 did in Kibler. In contrast, all breeding lines showed significant differences between locations for linolenic acid concentration, except for R07-8292. The different response of R07-8292 compared to the other lines may have caused the significant location by breeding line interaction for oleic and linolenic acid concentrations. Differences in genetic background and breeding history of the breeding lines studied had a larger effect on seed organic components than the environment (locations) where the lines were evaluated. It is possible that the differences in soil properties and leaf inorganic nutrient concentration between the locations did not contribute significantly to variation in organic seed composition. Maybe deficient or extremely high levels of chemical elements in the soil are needed to measure considerable differences.

Five breeding lines did not fully express their seed traits as expected (Table 6). High protein lines, R05-1772 and R05-1415, high oil lines, R02-6268F and R05-655, and high oleic line, R07-8292, showed lower protein, oil, and oleic acid concentrations than what is usually expected for competitive high protein (>45 mg g^{-1}), high oil lines (>22 mg g^{-1}), and high oleic (>45 mg g^{-1}) lines, respectively. Jaureguy et al. (2012) reported that R05-1772 and R05-1415 showed protein

concentration above 44 mg g^{-1} when grown at the same locations as this study, but in 2010. In Jaureguy et al. (2012), R07-8292 exhibited oleic acid above 45 mg g^{-1} at both locations when planted before June. Therefore, the differences in these traits between years may be related to other factors than soil properties. Temperature and water deficit stress affect seed composition (Dornbos and Mullen, 1992; Piper and Boote, 1999) and may be probably the reason for the differences observed for these traits between years. On the other hand, R02-6268F and R05-655 were still below the oil range in 2010 (Jaureguy et al., 2012), which indicated that these two high oil lines, although they may have high oil pedigree, may not be appropriate for the production of high oil soybeans in the locations studied. Possibly, other soybean production areas such as the Delta regions with warmer temperatures in Arkansas may potentially maximize the expression of the oil trait in these two breeding lines.

All seed chemical elements, except for Ca, showed no breeding line by location interaction (Table 5).The lack of interaction and significant differences among both locations and breeding lines suggested that seed inorganic components responded to the effect of the environment, but without significant changes in breeding line ranking. Alternatively, lack of genetic variation may also explain these differences.

Overall, soybeans had greater linolenic acid, P, K, Ca, Mg, and B concentrations at Kibler, but greater oleic acid, sucrose, stachyose, S and Zn concentrations at Fayetteville (Table 7). Differences in leaf chemical elements between locations were not reflected in seed chemical elements. For example, P, K, Ca, S, and Cu concentrations did differ between locations in the leaf, but, except for Cu, they were significantly different in the seed. Further, Fe and Mn showed significant differences between locations in the leaf, but exhibited similar concentrations in the seed. Seed-to-leaf enrichment ratios (see Tyler and Zohen, 1998) showed that plants at

Fayetteville had greater seed P and Mg relative to the leaf than plants grown at Kibler (Figure 1). Similarly, plants grown at Kibler had greater seed Ca relative to the leaf than plants grown at Fayetteville. Because leaf chemical element concentrations were within the medium-optimum range at both locations, it was proposed that the differences observed in seed enrichment ratios may have been caused by different rates of chemical element remobilization/uptake between locations from the time plants were sampled until maturity. The lack of direct relationships could mean that soil had no impact on leaf chemical elements and seed traits, or all soil properties measured were adequate, in other words no deficiencies or excesses. Future research should involve experiments with minerals that showed the largest contrast between leaf and seed concentrations and applied at deficient, optimum, and excessive levels to the growing plant.

Correlation analysis

The interpretation of correlations between soil and leaf nutrients, and seed composition has to be approached cautiously and considering the fact that two variables are correlated does not necessarily imply causality. This becomes important for chemical elements (Slipcevic et al., 1993; Gibson and Mullen, 2001), and to a greater extent, for organic seed components, which have long been shown to be greatly affected by moisture levels and temperature (Dornbos and Mullen, 1992; Piper and Boote, 1999) experienced by plants during the seed-fill period. In 2011, Kibler experienced significantly warmer temperatures than Fayetteville during the month of September (see Jaureguy et al., 2012), which coincides with the seed-fill period of the breeding lines used in this study, which may have possibly contributed to the differences observed in oleic and linolenic acid. Nonetheless, the correlations reported here can help complement the present understanding of factors including soil properties controlling/affecting seed composition in soybean.

Fayetteville showed greater number of significant correlations than Kibler, which suggested that soil properties and the response of breeding lines to the environment was more variable than when plants were grown at Kibler (Tables 8, 9,10,11,12,13). Fayetteville exhibited significant correlations between soil pH and several soil elements (Table 8), which indicated that the low pH at Fayetteville may have had a greater influence on the concentration of extractable chemical elements in the soil than nearly neutral soil pH observed at Kibler (Table 11).

In general, an except for a few cases, soil and leaf variables were not correlated with organic seed components, which suggested that the soil and the leaf variable studied did not covary significantly with organic seed components (Tables 9, 10, 12, 13). Several other studies have also failed to show significant association between inorganic and organic seed components. Boswell and Worthington (1971) studied the effects of varying amounts of soil B and soil Mn on seed protein, oil, and fatty acids in a field experiment. Soil Mn and soil B had only a slight effect on protein concentration, but differences were not significant. Oil and fatty acids were not affected by the treatments. Similarly, Heenan and Campbell (1980) observed no effect of Mn in the growing medium on protein and oil of two cultivars grown in the greenhouse. Wilson (1982) reported that deficient levels of soil Mn increased seed linoleic, linolenic, saturated fatty acids and protein, but decreased oil and oleic acid concentrations. Interestingly, these effects were absent when plants were above the deficiency levels (15 mg kg^{-1}). Ham et al. (1975) studied the effect of S fertilizer on seed protein and fatty acids and reported no effect of S on these two seed components. Similar results were observed for protein and oil measured in soybeans grown at different S fertilizer rates in Missouri (Brown et al., 1981), Kansas (Sweeney and Granade, 1993) and Iowa (Haq and Mallarino, 2005), and increasing levels of macronutrients N, P, K, Ca, and Mg in soybeans grown in hydroponic conditions (Harper, 1971). On the other hand, few studies

have shown highly significant relationships between soil or leaf chemical elements and seed organic composition. Gaudou and Arrivets (1983) studied the effect of soil P, K, and N on soybean seed organic components and showed that P increased protein and oleic acid, whereas soil K increased oil and linolenic acid, but decreased protein concentrations. Bellaloui et al. (2009a; 2010) studied the effect of foliar B application on soybean seed composition and demonstrated a significant increase in protein and oleic acid, but a decrease in oil and linolenic acid concentrations. In a different study, Bellaloui et al. (2009b) observed a significant correlation between K, B, and Zn in the soil and seed protein and oleic acid for a high-yield cultivar grown at one location for two consecutive years. The lack of agreement among the studies described here suggests that cultivars (high yield, specialty), environments (temperature, soil), and methods (field, greenhouse) may have accounted for the observed variation. It appears that if producers want to attain different levels of organic seed components, fertilization with chemical elements would not be a recommended practice. Adjusting planting date maybe an easier and more effective alternative. Planting early has been shown to increase oil and oleic acid concentration (Wilcox and Cavins, 1992; Kane et al., 1997), whereas late planting tends to increase sucrose (Bellaloui et al., 2011; Jaureguy et al., 2012).

Soil and leaf concentration of K, Mg, Fe, Zn, and Cu were correlated in Fayetteville, whereas only soil and leaf concentration of Mn were correlated in Kibler. The fact that Kibler exhibited, overall, very satisfactory soil properties for soybean growth and more optimum than Fayetteville may explain the lack of covariation, and therefore correlation, between soil, leaf and seed chemical element concentrations in Kibler as compared to Fayetteville. Variation in the concentration of seed chemical elements was, in general, not explained by variation in soil or leaf element concentration (Table 14). The only exception was seed Zn concentration in

Fayetteville, in which 47% if the variation was explained by soil leaf Zn (Table 14). These results suggest that in Fayetteville, leaf Zn concentration may be an adequate predictor or seed Zn concentration.

In this study it was shown that although they had a different breeding history, all the specialty soybean breeding lines were able take up, transport, and increase leaf chemical element concentration to adequate levels before the reproductive period. Differences in leaf minerals between locations were not translated to the seed. Other factors such as temperature during seed-fill may have overridden potential association between leaf minerals and seed components. Altering fertilization practices may not result in changes in seed composition. Results presented in this study may be used as a starting point for research aimed to understand which mechanisms play a relevant role in seed composition, and ultimately develop fertilization and management practices that improve crop seed quality (Cakmak et al., 2010).

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Table 1. Seed traits of soybean lines used in the planting date and delayed harvest study.

Genotype†	Trait
R05-1772‡	high protein
R05-1415‡	high protein
R05-655	high oil
R02-6268F	high oil
R07-8292	high-oleic acid
R05-5346	low linolenic acid
R06-814	low saturated fatty acids (stearic + palmitic acid)
R07-2000	high sucrose, low stachyose, high inorganic phosphorus
AG5605	high yield

† all lines, except AG5605 which is property of Asgrow Seed Company, were developed at the University of Arkansas soybean breeding program.

‡ see Chen et al. (2008) and Chen et al. (2011) for more details on these lines.

Table 2. Physical and chemical properties of the soils at the experiment sites where the study was conducted.

Soil properties†	Fayetteville	Kibler	Ratio‡	F test§	Low range¶
texture	Silt loam	Silt loam	-	-	-
clay %	9	23	2.6	***	-
sand %	38	24	0.6	***	-
Silt %	52	53	1.0	NS	-
pH	5.3	7.1	1.4	***	-
EC (µmhos cm^{-1})	163	106	0.7	*	-
P	44	47	1.1	NS	16-25
K	81	152	1.9	**	61-90
Ca	682	1839	2.7	**	<400
Mg	51	382	7.5	**	<30
S	15	8	0.5	**	<10
Fe	142.2	129.5	0.91	NS	-
Mn	81.6	117.6	1.44	**	<40
Zn	0.8	2.7	3.44	**	<1.6-3
Cu	0.3	2.0	7.63	**	<1
B	0.3	0.6	2.02	**	<0.025#

† EC = electrical conductivity; all minerals expressed in mg kg^{-1}.

‡ ratio between the seed component concentration observed at Kibler and the concentration observed at Fayetteville.

§ from the location effect in ANOVA.

¶ the low range values were obtained, when available, from the University of Arkansas Soil Test report.

NS not significant at the 0.05 probability level.

* significant at 0.05 level.

** significant at 0.01 level.

*** significant at 0.001 level.

data from Minarik and Shive 1939.

Table 3. Analysis of variance of leaf chemical element concentrations for breeding lines with modified seed composition grown at two Arkansas locations (Fayetteville and Kibler) in 2011.

Source of effects	Dfn†	Dfd‡	P§	K	Ca	Mg	S	Fe	Mn	Zn	Cu	B
Location (Loc)	1	4	NS	NS	NS	***	NS	**	***	*	NS	***
Breeding line (Bl)	8	32	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Loc x Bl	8	32	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

† degrees of freedom of numerator in F test.

‡ degrees of freedom of denominator in F test.

§ P, K, Ca, Mg, S in %; Fe, Mn, Zn, Cu and B in mg kg^{-1}

NS not significant at the 0.05 probability level.

* significant at 0.05 level.

** significant at 0.01 level.

*** significant at 0.001 level.

Table 4. Leaf chemical element concentrations of breeding lines (combined) with modified seed composition grown at two Arkansas locations (Fayetteville and Kibler) in 2011.

Mineral†	Fayetteville	Kibler	Ratio‡	F test§	Optimum range¶
P	0.3	0.4	1.14	NS	0.1-0.5
K	2.1	2.3	1.05	NS	0.5-0.6
Ca	1.1	1.1	0.95	NS	0.2-1
Mg	0.3	0.4	1.43	***	0.1-0.4
S	0.3	0.3	0.98	NS	0.25-0.3
Fe	126	179	1.42	**	50-250
Mn	61	40	0.66	**	20-500
Zn	34	43	1.27	*	25-150
Cu	10	9	0.99	NS	5-20
B	27	42	1.56	***	20-60

† P, K, Ca, Mg, S in %. Fe, Mn, Zn, Ci, B in mg kg^{-1}.

‡ ratio between the seed component concentration observed at Kibler and the concentration observed at Fayetteville.

§ from the location effect in ANOVA.

¶ from Marschner (1997).

NS not significant at the 0.05 probability level.

* significant at 0.05 level.

** significant at 0.01 level.

*** significant at 0.001 level.

Table 5. Analysis of variance of seed organic and inorganic components for breeding lines with modified seed composition grown at two Arkansas locations (Fayetteville and Kibler) in 2011.

Source of effects†	Pro‡	Oil	Ole	Lin	Sats	Suc	Sta	Pi	P	K	Ca	Mg	S	Fe	Mn	Zn	Cu	B
Location (Loc)	NS	NS	**	***	NS	**	**	NS	**	***	***	***	**	NS	NS	**	NS	***
Breeding line (Bl)	***	***	***	***	***	***	***	***	***	***	***	***	***	**	NS	***	NS	*
Loc x Bl	NS	NS	***	***	NS	NS	NS	NS	NS	NS	**	NS	NS	NS	NS	NS	NS	NS

† degrees of freedom of numerator in F test: Loc = 1; Bl = 8, Loc x Bl = 8. Degrees of freedom of denominator in F test: Loc = 4; Bl = 25, Loc x Bl = 25.

‡ Pro = protein concentration (mg g^{-1}); Oil concentration in mg g^{-1}; Ole = oleic acid concentration (mg g^{-1}); Lin = linolenic acid concentration (mg g^{-1}); Sats = saturated fatty acids (palmitic + stearic acids) (mg g^{-1}); Suc = sucrose concentration (mg g^{-1}); Sta = stachyose concentration (mg g^{-1}); Pi = inorganic phosphorus concentration (µg g^{-1}). From P to S in mg g^{-1}; from Fe to Zn in mg kg^{-1}.

NS, not significant at the 0.05 probability level.

* significant at 0.05 level.

** significant at 0.01 level.

*** significant at 0.001 level.

Table 6. Seed organic and inorganic component concentrations for breeding lines with modified seed composition grown at two Arkansas locations (combined) in 2011.

Breeding line	Pro†	Oil	Ole	Lin	Sats	Suc	Sta	Pi	P	K	Ca	Mg	S	Fe	Mn	Zn	Cu	B
R05-1772	382	178	203	65	146	47	44	159	7.6	21.1	2.5	2.9	3.8	56	33	41	14	31
R05-1415	390	179	199	63	152	47	40	177	7.7	21.6	2.4	2.7	3.8	61	33	47	16	30
R05-655	349	195	237	56	144	53	47	175	6.8	19.6	2.9	2.6	3.4	52	30	40	14	29
R02-6268F	333	198	257	54	147	56	44	192	6.3	19.4	2.7	2.3	3.3	48	29	39	15	32
R07-8292	371	169	322	39	141	59	46	215	6.9	18.0	2.5	2.3	3.4	54	28	43	16	31
R05-5346	353	190	243	32	144	55	44	183	6.2	20.1	2.1	2.4	3.4	55	28	38	16	33
R06-814	377	177	255	41	80	46	45	217	6.8	20.6	2.6	2.5	3.6	54	31	41	16	28
R07-2000	371	182	252	59	138	90	2	1733	6.7	19.8	2.5	2.4	3.5	58	29	43	15	29
AG5605	364	189	240	53	144	45	47	187	6.6	19.5	3.5	2.6	3.3	61	32	47	18	33
LSD‡	10	5	12	4	6	4	2	60	0.2	0.5	0.2	0.1	0.1	4.3	3.7	3.0	1.7	2.2

† pro = protein concentration (mg g^{-1}); Oil concentration in mg g^{-1}; Ole = oleic acid concentration (mg g^{-1}); Lin = linolenic acid concentration (mg g^{-1}); Sats = saturated fatty acids (palmitic + stearic acids) (mg g^{-1}); Suc = sucrose concentration (mg g^{-1}); Sta = stachyose concentration (mg g^{-1}); Pi = inorganic phosphorus concentration (µg g^{-1}). From P to S in mg g^{-1}; from Fe to B in mg kg^{-1}.

‡ least significant difference at 0.05 probability level.

Table 7. Seed chemical element concentrations of breeding lines (combined) with modified seed composition grown at two Arkansas locations (Fayetteville and Kibler) in 2011.

Seed component†	Fayetteville	Kibler	Ratio‡	F test§
Pro	362	369	1.02	NS
Oil	183	185	1.01	NS
Ole	255	235	0.92	*
Lin	41	61	1.48	*
Sats	138	137	1.00	NS
Suc	61	49	0.81	*
Sta	42	38	0.90	*
Pi	375	345	0.92	NS
P	6.7	7.0	1.06	*
K	19.2	20.7	1.08	*
Ca	2.2	3.0	1.36	*
Mg	2.4	2.7	1.11	*
S	3.6	3.4	0.94	*
Fe	55	56	1.02	NS
Mn	31	29	0.94	NS
Zn	46	38	0.83	*
Cu	15	16	1.07	NS
B	24	37	1.52	*

† Pro = protein concentration (mg g^{-1}); Oil concentration in mg g^{-1}; Ole = oleic acid concentration (mg g^{-1}); Lin = linolenic acid concentration (mg g^{-1}); Sats = saturated fatty acids (palmitic + stearic acids) (mg g^{-1}); Suc = sucrose concentration (mg g^{-1}); Sta = stachyose concentration (mg g^{-1}); Pi = inorganic phosphorus concentration (µg g^{-1}). From P to S in mg g^{-1}; from Fe to B in mg kg^{-1}.

‡ ratio between the seed component concentration observed at Kibler and the concentration observed at Fayetteville.

§ location effect from ANOVA.

* significant at the 0.05 probability level.

Table 8. Pearson correlation coefficients (n = 27) between soil properties and leaf chemical element concentrations in nine soybean breeding lines grown in Fayetteville, AR in 2011 with three replications.

	Leaf P	Leaf K	Leaf Ca	Leaf Mg	Leaf S	Leaf Fe	Leaf Mn	Leaf Zn	Leaf Cu	Leaf B
clay %	-0.20	0.01	0.04	-0.17	-0.25	-0.14	-0.25	-0.21	0.00	0.21
sand %	-0.10	-0.23	0.48	0.19	0.03	0.13	0.22	-0.08	-0.07	-0.27
silt %	0.27	0.23	-0.41	-0.03	0.16	-0.14	0.01	0.24	0.01	0.12
EC†	-0.18	0.09	-0.07	-0.31	-0.08	-0.25	0.23	-0.11	-0.01	0.26
Soil-P	0.10	-0.06	-0.27	-0.19	-0.20	-0.39*	0.35	0.36	-0.20	0.64***
Soil-K	0.25	0.68***	-0.23	0.06	0.20	-0.10	-0.25	-0.13	0.26	-0.16
Soil-Ca	0.63***	0.32	-0.32	0.29	0.36	-0.45*	-0.37	0.67***	-0.52**	-0.14
Soil-Mg	0.65***	0.50**	-0.38	0.42*	0.47*	-0.38	-0.37	0.62**	-0.36	-0.21
Soil-S	-0.08	0.18	-0.09	-0.15	-0.09	-0.29	0.27	0.14	-0.06	0.48*
Soil-Fe	0.54**	0.00	-0.30	0.24	0.20	-0.55**	-0.01	0.75***	-0.52**	0.08
Soil-Mn	0.12	0.45*	-0.28	-0.14	-0.09	-0.30	0.06	0.00	0.14	0.25
Soil-Zn	0.60**	0.26	-0.39*	0.19	0.15	-0.36	-0.12	0.65***	-0.25	0.24
Soil-Cu	0.37	0.17	-0.12	0.11	0.14	-0.39*	0.02	0.50**	-0.40*	0.27
Soil- B	0.06	-0.09	0.04	-0.05	-0.17	-0.19	-0.17	0.04	-0.38*	-0.06

† electrical conductivity.

* significant at 0.05 level.

** significant at 0.01 level.

*** significant at 0.001 level.

Table 9. Pearson correlation coefficients (n = 27) between seed component concentrations and soil properties in nine soybean breeding lines grown in Fayetteville, AR in 2011 with three replications.

	clay %	sand %	silt %	pH	EC‡	Soil P	Soil K	Soil Ca	Soil Mg	Soil S	Soil Fe	Soil Mn	Soil Zn	Soil Cu	Soil B
Pro†	0.13	0.03	-0.08	0.04	-0.02	0.11	-0.18	-0.06	-0.18	0.20	0.26	0.14	-0.01	-0.10	0.13
Oil	0.02	0.04	-0.03	-0.01	0.07	-0.11	-0.05	0.02	0.06	-0.32	-0.18	-0.09	-0.09	-0.05	-0.02
Ole	0.01	0.26	-0.28	0.09	-0.10	-0.25	0.01	-0.05	-0.11	0.01	-0.25	-0.11	-0.03	0.05	-0.27
Lin	0.23	0.02	-0.17	0.16	-0.33	-0.10	-0.40	0.16	0.16	-0.09	0.23	-0.30	-0.10	0.08	0.42
Sats	-0.02	-0.13	0.11	-0.03	0.14	0.26	-0.15	0.14	0.17	0.21	0.19	0.07	0.19	0.15	0.06
Suc	0.20	0.03	-0.14	0.37	-0.24	-0.01	-0.03	0.31	0.46*	-0.07	0.38	-0.43	0.16	0.35	0.37
Sta	-0.30	-0.10	0.25	-0.40	0.34	0.10	0.21	-0.21	-0.32	0.12	-0.39	0.50*	-0.08	-0.21	-0.40
Pi	0.31	0.07	-0.23	0.35	-0.28	-0.07	-0.20	0.21	0.28	-0.07	0.37	-0.46*	0.09	0.25	0.39
Seed-P	0.07	-0.19	0.13	-0.05	0.00	0.34	-0.28	0.09	0.01	0.21	0.33	0.23	0.17	0.07	0.25
Seed-K	0.02	-0.03	0.00	-0.02	-0.06	-0.04	0.07	-0.25	-0.25	-0.21	-0.09	0.18	-0.18	-0.40	0.13
Seed-Ca	0.21	0.08	-0.23	-0.02	0.02	0.01	-0.48*	0.16	0.02	0.24	0.03	-0.08	-0.03	0.24	0.00
Seed-Mg	0.03	-0.06	0.04	-0.03	-0.02	0.01	-0.20	-0.05	-0.04	-0.12	0.13	0.10	-0.21	-0.28	0.35
Seed-S	0.06	0.15	-0.17	0.15	-0.24	-0.23	-0.11	-0.21	-0.20	-0.31	0.00	0.00	-0.25	-0.43	0.20
Seed-Fe	0.02	0.01	0.05	0.40	-0.20	0.12	-0.30	0.21	0.37	-0.10	0.52*	-0.20	0.11	0.00	0.12
Seed-Mn	0.02	0.25	-0.26	-0.51*	0.10	-0.34	0.20	-0.76***	-0.78***	-0.03	-0.69**	0.33	-0.72***	-0.60**	-0.15
Seed-Zn	0.14	0.12	-0.15	0.26	-0.24	0.25	-0.51	0.20	0.21	0.20	0.49*	-0.03	0.23	0.21	0.05
Seed-Cu	0.42	-0.04	-0.22	0.20	-0.26	-0.19	-0.29	-0.23	-0.11	0.04	-0.18	-0.01	-0.38	-0.23	-0.21
Seed-B	-0.27	-0.20	0.38	-0.03	0.20	0.75***	0.21	0.39	0.54*	0.35	0.62**	0.35	0.61**	0.57**	0.18

† Pro = protein concentration; Oil concentration; Ole = oleic acid concentration; Lin = linolenic acid; Sats = saturated fatty acids concentration; Suc = sucrose concentration; Sta = stachyose concentration; Pi = inorganic phosphorus concentration.

‡ electrical conductivity.

* significant at 0.05 level.

** significant at 0.01 level.

*** significant at 0.001 level.

Table 10. Pearson correlation coefficients (n = 27) between seed component concentrations and leaf chemical element concentrations in nine soybean breeding lines grown in Fayetteville, AR in 2011 with three replications.

	Leaf P	Leaf K	Leaf Ca	Leaf Mg	Leaf S	Leaf Fe	Leaf Mn	Leaf Zn	Leaf Cu	Leaf B
Prot†	0.21	0.10	-0.27	-0.18	-0.53*	-0.67**	-0.06	0.17	-0.29	0.05
Oil	-0.12	-0.37	0.15	0.31	0.48*	0.34	-0.05	-0.15	0.05	-0.05
Ole	-0.10	0.26	0.15	0.04	0.06	0.35	0.16	-0.06	0.42	0.03
Lin	0.09	-0.36	0.33	0.31	-0.09	-0.04	-0.01	0.17	-0.62**	-0.05
Sats	0.02	-0.33	0.21	0.00	0.32	-0.03	0.32	0.36	-0.29	0.32
Suc	0.22	0.22	0.12	0.25	0.23	-0.03	-0.13	0.20	-0.10	0.10
Sta	-0.25	-0.12	-0.12	-0.26	-0.05	0.21	0.22	-0.17	0.25	-0.08
Pi	0.22	0.11	0.09	0.24	0.01	-0.17	-0.19	0.13	-0.17	0.14
Seed-P	0.31	-0.10	-0.13	-0.04	-0.32	-0.42	0.13	0.42	-0.50*	0.24
Seed-K	0.07	0.07	-0.16	-0.16	-0.21	-0.18	-0.29	-0.12	-0.16	-0.16
Seed-Ca	-0.22	-0.49*	0.17	0.43	-0.36	0.12	0.35	0.11	-0.06	0.30
Seed-Mg	0.12	-0.22	0.00	0.16	-0.24	-0.30	0.03	0.12	-0.42	-0.14
Seed-S	0.17	0.08	-0.03	-0.05	-0.17	-0.31	-0.24	0.00	-0.29	-0.28
Seed-Fe	0.49*	0.14	-0.35	0.33	-0.03	-0.70***	-0.12	0.58**	-0.35	0.09
Seed-Mn	-0.54*	-0.25	0.46*	0.00	-0.15	0.26	0.40	-0.60**	0.33	-0.16
Seed-Zn	0.37	-0.13	-0.16	0.30	-0.25	-0.55**	0.21	0.68**	-0.44*	0.38
Seed-Cu	-0.18	0.05	-0.23	0.26	-0.09	0.00	0.12	0.10	0.43	0.26
Seed-B	0.39	0.18	-0.24	0.02	0.02	-0.30	0.25	0.64**	-0.34	0.37

* significant at 0.05 level.

** significant at 0.01 level.

*** significant at 0.001 level.

† Pro = protein concentration; Oil concentration; Ole = oleic acid concentration; Lin = linolenic acid; Sats = saturated fatty acids concentration; Suc = sucrose concentration; Sta = stachyose concentration; Pi = inorganic phosphorus concentration.

Table 11. Pearson correlation coefficients (n = 27) between soil properties and leaf chemical element concentrations in nine soybean breeding lines grown in Kibler, AR in 2011 with three replications.

	Leaf P	Leaf K	Leaf Ca	Leaf Mg	Leaf S	Leaf Fe	Leaf Mn	Leaf Zn	Leaf Cu	Leaf B
clay %	-0.07	0.18	0.05	-0.25	0.17	-0.34	-0.33	0.16	0.16	-0.14
sand %	-0.19	-0.24	0.21	0.28	-0.10	0.28	0.27	-0.20	-0.17	0.04
silt %	0.30	0.08	-0.28	-0.03	-0.05	0.04	0.06	0.06	0.02	0.10
pH	-0.29	-0.18	-0.05	0.12	0.22	-0.37	0.08	-0.01	0.06	0.07
EC†	0.11	-0.03	0.26	-0.01	0.34	-0.13	-0.05	0.20	0.26	-0.17
Soil-P	0.33	0.35	-0.06	-0.23	0.03	0.07	-0.19	0.23	0.17	-0.18
Soil-K	0.33	0.28	-0.07	-0.27	0.34	-0.29	-0.42*	0.42*	0.41*	-0.27
Soil-Ca	0.15	-0.07	0.09	-0.04	0.55	-0.41	-0.26	0.48	0.51	-0.07
Soil-Mg	0.25	0.00	0.03	-0.05	0.60***	-0.50**	-0.35	0.53**	0.58**	-0.11
Soil-S	0.38*	0.21	-0.15	-0.13	0.00	0.10	-0.14	0.18	0.22	-0.23
Soil-Fe	0.16	0.40*	-0.15	-0.24	-0.36	0.35	-0.20	-0.22	-0.22	-0.31
Soil-Mn	0.40*	0.25	-0.12	-0.26	0.34	-0.24	-0.45*	0.46	0.43*	-0.26
Soil-Zn	0.41*	0.38	-0.14	-0.13	0.01	0.10	-0.18	0.10	0.07	-0.13
Soil-Cu	0.27	0.09	0.04	-0.11	0.45*	-0.23	-0.31	0.43	0.44*	-0.21
Soil- B	0.46	0.10	0.00	0.01	0.57**	-0.40*	-0.22	0.45*	0.57*	-0.18

* significant at 0.05 level.

** significant at 0.01 level.

*** significant at 0.001 level.

† electrical conductivity.

Table 12. Pearson correlation coefficients (n = 27) between seed component concentrations and soil properties in nine soybean breeding lines grown in Kibler, AR in 2011 with three replications.

	clay %	sand %	silt %	pH	EC‡	Soil P	Soil K	Soil Ca	Soil Mg	Soil S	Soil Fe	Soil Mn	Soil Zn	Soil Cu	Soil B
Pro†	-0.07	0.30	-0.28	0.01	0.20	-0.20	-0.25	-0.07	-0.09	-0.20	-0.06	-0.16	-0.07	-0.18	-0.08
Oil	0.06	-0.31	0.29	-0.13	-0.10	0.28	0.33	0.12	0.16	0.35	0.18	0.31	0.17	0.33	0.17
Ole	-0.01	-0.15	0.20	0.16	-0.29	-0.24	-0.24	-0.28	-0.27	-0.22	-0.07	-0.30	-0.21	-0.40*	-0.31
Lin	-0.02	0.21	-0.24	0.06	0.08	0.07	0.00	0.01	0.02	0.06	0.17	0.11	0.14	0.18	0.17
Sats	-0.17	0.05	0.14	0.08	-0.04	-0.14	-0.10	-0.06	-0.05	-0.11	-0.10	0.05	0.05	-0.05	-0.10
Suc	0.18	-0.34	0.15	-0.08	-0.05	0.01	0.03	-0.04	-0.02	0.01	0.11	0.08	0.01	0.02	-0.02
Sta	-0.22	0.35	-0.12	0.06	0.01	0.00	-0.06	-0.05	-0.12	-0.07	-0.10	-0.18	-0.11	-0.11	-0.16
Pi	0.14	-0.28	0.12	-0.07	-0.02	-0.05	-0.01	-0.01	0.05	0.04	0.10	0.10	0.04	0.07	0.08
Seed-P	-0.03	0.26	-0.25	-0.03	0.19	-0.02	-0.17	-0.08	-0.11	-0.13	0.00	-0.11	0.03	-0.15	-0.06
Seed-K	0.06	0.06	-0.14	-0.24	0.34	0.17	0.11	0.23	0.23	0.12	-0.01	0.26	0.20	0.28	0.28
Seed-Ca	-0.36	0.35	0.02	0.03	-0.14	-0.27	-0.26	-0.16	-0.12	-0.07	-0.04	-0.17	-0.07	-0.08	-0.12
Seed-Mg	-0.14	0.37	-0.26	-0.08	0.16	0.15	0.04	0.06	0.04	0.12	0.07	0.03	0.16	0.15	0.16
Seed-S	0.03	0.11	-0.16	-0.21	0.21	0.00	-0.18	-0.09	-0.10	-0.05	0.07	0.01	0.12	-0.11	-0.04
Seed-Fe	-0.42*	0.22	0.25	0.01	0.03	-0.24	-0.40	-0.32	-0.27	-0.09	0.00	-0.31	0.06	-0.47*	-0.26
Seed-Mn	-0.32	0.24	0.10	-0.17	-0.01	-0.17	-0.32	-0.22	-0.16	0.02	0.09	-0.10	0.20	-0.18	-0.06
Seed-Zn	-0.36	-0.08	0.50**	-0.27	-0.01	0.18	-0.13	-0.43	-0.32	0.31	0.48*	-0.06	0.47*	-0.25	-0.14
Seed-Cu	-0.22	-0.13	0.42*	-0.22	0.11	0.20	0.09	-0.14	-0.09	0.33	0.32	-0.01	0.24	-0.01	0.13
Seed-B	-0.42*	0.18	0.31	-0.13	-0.18	-0.02	-0.20	-0.22	-0.19	0.04	0.02	-0.12	0.25	-0.21	-0.17

* significant at 0.05 level.

** significant at 0.01 level.

*** significant at 0.001 level.

† Pro = protein concentration; Oil concentration; Ole = oleic acid concentration; Lin = linolenic acid; Sats = saturated fatty acids concentration; Suc = sucrose concentration; Sta = stachyose concentration; Pi = inorganic phosphorus concentration.

‡ electrical conductivity.

Table 13. Pearson correlations (n = 27) between seed component concentrations and leaf chemical element concentrations in nine soybean breeding lines grown in Kibler, AR in 2011 with three replications.

	Leaf P	Leaf K	Leaf Ca	Leaf Mg	Leaf S	Leaf Fe	Leaf Mn	Leaf Zn	Leaf Cu	Leaf B
Prot†	0.20	0.13	-0.07	-0.03	0.06	-0.09	-0.18	0.05	0.24	0.01
Oil	-0.18	-0.24	0.01	0.05	-0.11	0.18	0.17	-0.09	-0.16	-0.09
Ole	-0.14	0.10	-0.11	-0.03	-0.19	-0.11	0.09	-0.11	-0.26	0.24
Lin	0.15	0.13	-0.17	0.00	0.06	0.07	-0.03	0.03	0.09	-0.17
Sats	0.15	-0.09	-0.36	-0.05	0.17	-0.28	0.05	-0.02	0.01	0.13
Suc	-0.02	0.16	-0.44*	-0.26	-0.23	0.06	-0.04	-0.05	-0.16	0.26
Sta	-0.17	-0.06	0.49**	0.16	-0.04	0.16	0.10	-0.07	-0.12	-0.24
Pi	0.08	0.04	-0.49*	-0.20	-0.05	-0.04	-0.06	0.00	0.02	0.22
Seed-P	0.16	0.35	0.22	0.00	-0.01	-0.02	-0.21	0.06	0.07	-0.17
Seed-K	0.31	-0.12	0.11	0.04	0.27	-0.03	-0.03	0.15	0.34	-0.09
Seed-Ca	-0.03	-0.29	-0.10	0.18	0.08	0.04	0.27	-0.08	-0.05	-0.08
Seed-Mg	0.07	0.06	0.22	0.09	0.10	0.21	-0.04	0.13	0.10	-0.15
Seed-S	0.19	0.23	0.22	-0.02	0.01	0.01	-0.17	0.05	0.08	-0.11
Seed-Fe	0.23	-0.15	-0.25	0.13	0.07	-0.21	0.09	-0.12	0.10	0.01
Seed-Mn	0.26	-0.16	-0.10	0.07	0.23	-0.07	0.01	-0.01	0.20	-0.07
Seed-Zn	0.34	0.24	-0.17	-0.03	0.01	-0.11	-0.11	-0.14	0.03	-0.18
Seed-Cu	0.20	-0.10	0.19	0.22	-0.09	0.09	0.12	-0.24	-0.05	-0.11
Seed-B	0.14	-0.29	0.07	0.27	0.32	-0.03	0.25	-0.04	0.04	0.12

* significant at 0.05 level.

** significant at 0.01 level.

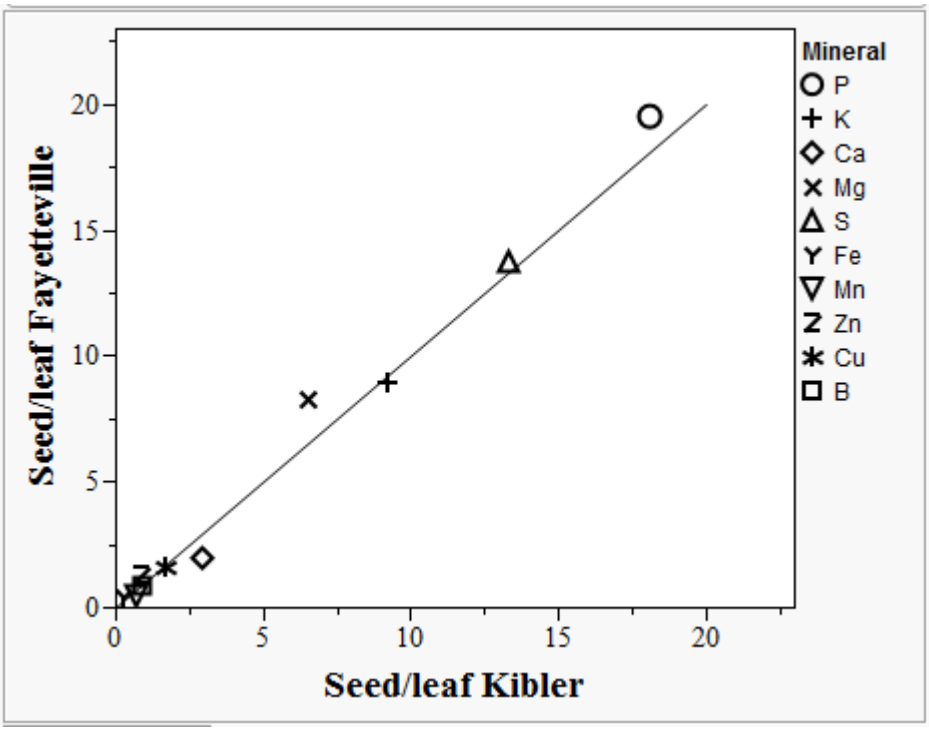
*** significant at 0.001 level.

† Pro = protein concentration; Oil concentration; Ole = oleic acid concentration; Lin = linolenic acid; Sats = saturated fatty acids concentration; Suc = sucrose concentration; Sta = stachyose concentration; Pi = inorganic phosphorus concentration.

Table 14. Sum of squares for each of the factors of the model: soil element concentration (Soil) + leaf element concentration (Leaf) + error = seed element concentration, calculated as a measure of the contribution of soil element concentration and leaf element concentration to the variability observed in the seed element concentration in nine soybean breeding lines grown in Kibler, AR in 2011 with three replications.

	Seed P	**Seed K**	**Seed Ca**	**Seed Mg**	**Seed S**	**Seed Fe**	**Seed Mn**	**Seed Zn**	**Seed Cu**	**Seed B**
						Fayetteville				
Soil	0.63	0.07	0.08	0.01	0.20	4.42	18.14	27.34	0.11	7.60
Leaf	0.50	0.05	0.09	0.03	0.13	146.71	45.02	201.69	8.14	27.46
Error	5.66	18.86	2.03	0.81	0.77	317.77	424.30	194.76	50.51	151.70
Total	6.79	18.99	2.21	0.86	1.11	468.89	487.47	423.80	58.77	186.76
						Kibler				
Soil	0.06	1.10	0.14	0.00	0.00	4.91	2.08	105.06	0.03	4.49
Leaf	0.28	1.13	0.05	0.01	0.00	38.64	0.24	16.59	0.32	1.55
error	8.25	45.26	6.18	1.07	0.97	745.97	172.41	331.63	99.06	188.79
Total	8.59	47.48	6.37	1.08	0.97	789.52	174.72	453.28	99.41	194.83

Figure 1. Seed enrichment ratios (seed chemical element concentration/leaf chemical element concentration) of breeding lines (combined) with modified seed composition grown at two Arkansas locations (Fayetteville and Kibler) in 2011. The line represents equal enrichment ratios at both locations. Elements above the line have higher enrichment ratios at Fayetteville than at Kibler and vice versa.



Conclusion

The findings reported in this dissertation will help provide producers with high metabolizable energy environmentally friendly soybeans with adaptation to southern U.S. Future cultivars with modified seed composition may come with enhanced recommendations on management practices that will assist producers plan both their planting and harvest strategies. Thus, producers will be able to grow cultivars that will be more competitive in the market, increase their profits, and contribute to a better and sustainable agriculture.