The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled:

VIABILITY, VIGOR, AND FIELD PERFORMANCE OF A LOW PHYTIC ACID, HIGH PHYTASE ACTIVITY SOYBEAN LINE, CAPPA

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VIABILITY, VIGOR, AND FIELD PERFORMANCE OF A LOW PHYTIC ACID, HIGH PHYTASE ACTIVITY SOYBEAN LINE, CAPPA

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS……………………………………………………………….ii

LIST OF TABLES…………………………………………………………………...v

LIST OF FIGURES…………………………………………………………………vii

ABSTRACT………………………………………………………………………….ix

CHAPTER 1: LITERATURE REVIEW AND RATIONALE………………………...1

CHAPTER 2: MATERIALS AND METHODS……………………………………….25

CHAPTER 3: RESULTS AND DISCUSSION………………………………………35

CHAPTER 4: SUMMARY AND CONCLUSIONS…………………………………49

REFERENCES CITED……………………………………………………………….69

LIST OF TABLES

Table Page

1 Total percent emergence of soybean seedlings of four entries in Seedling Dry Mass Study in 2006 and 2007 at the Bradford Research and Extension Center, Columbia, MO................……………………………51

2 Mean seed weight, number of seeds per plant, and yield at R8 of four soybean entries in the Yield Component Study. Seeds were produced in 2006 and 2007 at the Bradford Research and Extension Center, Columbia, MO………………………………………….…………………52

3 Total available mean P, phytic acid (PA), and phytase activity of R8 seeds of four soybean entries in the Yield Component Study. Seeds were produced in 2006 and 2007 at the Bradford Research and Extension Center, Columbia, MO……………………………………………………..53

4 Fatty acid content of R8 seeds of four soybean entries in the Yield Component Study. Seeds were produced in 2006 and 2007 at the Bradford Research and Extension Center, Columbia, MO………..………….54

5 Macronutrient and micronutrient analysis of R8 seeds of four soybean entries in the Yield Component Study. Seeds were produced in 2006 at the Bradford Research and Extension Center, Columbia, MO…………...………55

6 Macronutrient and micronutrient analysis of R8 seeds of four soybean entries in the Yield Component Study. Seeds were produced in 2007 at the Bradford Research and Extension Center, Columbia, MO..…………………56

7 Total percent standard germination and germination following accelerated aging of four entries of soybean seed at the R8 stage. Seeds were produced at the Bradford Research and Extension Center, Columbia, MO in 2006 and 2007………………………………………………………...…………57

8 Vigor tests, viability tests, and field emergence tests were utilized to evaluate four soybean entries. Vigor of seed utilized for laboratory vigor testing was compared to field emergence of Seedling Dry Mass Study seed……………………………………..…………………………..58

9 Analysis of soil samples from field plots in 2006 and 2007 at Bradford Research and Extension Center, Columbia, MO….…………………………..59

10 Managements applied to two field studies at the Bradford Research and Extension Center, Columbia, MO, in 2006 and 2007………………………………………………………………..60

LIST OF FIGURES

Figure Page

1 Cumulative above ground seedling dry mass of four soybean entries grown at the Bradford Research and Extension Center, Columbia, MO in 2007. Seedlings were harvested daily for 14 days consecutively starting 8 days after planting (DAP)……………………………………………61

2 Cumulative above ground seedling dry mass of four soybean entries grown at the Bradford Research and Extension Center, Columbia, MO in 2007. Seedlings were harvested daily for 14 days consecutively starting 8 days after planting (DAP). The predictive growth for the 7 days following sampling (22-28 DAP) are given to illustrate growth potential of the entries. Predictive growth equations for entries listed below the figure……………………………………………………………62

3 Mean total plant weight, excluding roots, of four soybean entries on five weekly sampling dates commencing at R5 (72 DAP) and concluding at R7 (107 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2006. Regression equations for entries are listed below the figure……………………………..63

4 Total above ground vegetative weight of four soybean entries on six sampling dates commencing at R5 (72 DAP) and concluding at R7 (107 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2006. Regression equations for entries are listed below the figure……………………………………………………….64

5 Total reproductive weight of four soybean entries on five sampling dates commencing at R5 (72 DAP) and concluding at R7 (100 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2006. Regression equations for entries are listed below the figure…………………………………………………………..65

6 Total above ground weight of four soybean entries on five sampling dates commencing at R5 (70 DAP) and concluding at R7 (98 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2007. Regression equations for entries are listed below the figure…………………………………………………………66

7 Total above ground vegetative weight of four soybean entries on five sampling dates commencing at R5 (70 DAP) and concluding at R7 (98 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2007. Regression equations for entries are listed below the figure……………………………………………67

8 Total reproductive weight of four soybean entries on five sampling dates commencing at R5 (70 DAP) and concluding at R7 (98 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2007. Regression equations for entries are listed below the figure………………………………………………………………68

VIABILITY, VIGOR, AND FIELD PERFORMANCE OF A LOW PHYTIC ACID, HIGH PHYTASE ACTIVITY SOYBEAN LINE, CAPPA

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Abstract

Plants store inorganic phosphate (P_i) in seeds as phytic acid (PA). Phytic acid chelates essential macro and micronutrients in developing seeds forming phytate, which is nutritionally unavailable to monogastric livestock. Composition changes to lower the PA content of seeds may affect seed viability and seedling performance. This project was conducted in 2006 and 2007 to test the viability, vigor, and field performance of a high phytase activity, transgenic soybean line, CAPPA compared to its progenitor, Jack. CAPPA seeds contain increased phytase activity, a >90% reduction in PA, and a concomitant increase in P_i. A seedling dry mass study measured dry mass gain for each entry from 8 days after planting (DAP) through 21 DAP. A yield component study measured the vegetative and reproductive dry mass gain for each entry from the R5-R8 stages. Laboratory vigor experiments involving standard germination, accelerated aging, cold test, electrical conductivity, and tetrazolium testing were conducted. Field emergence of all entries was low in 2006 and 2007. There was no significant difference in field emergence between CAPPA and Jack in 2006, but in 2007 Jack field emergence was significantly higher than CAPPA. There was no significant difference between CAPPA and Jack in seedling dry mass 8-21 DAP. Jack vegetative dry mass was

significantly greater than CAPPA in 2006 and 2007, but CAPPA seeds per plant, seed weight, and yield did not differ significantly from Jack over the two study years. There was no significant difference between Jack and CAPPA standard germination. Germination following accelerated aging was low but did not differ significantly between Jack and CAPPA. Jack seed germination was low following cold testing, but was significantly higher than CAPPA. Tetrazolium testing indicated no significant difference in viability between Jack and CAPPA. Electrolyte leakage of both entries was high, but did not differ significantly. CAPPA vigor appears to be lower than Jack, but due to high variability in the field environment over the study years, field emergence results are not conclusive. Further research is needed to indicate CAPPA's potential as a commercially released low PA soybean line. Emerged CAPPA plants grew to be competitive, high yielding plants.

CHAPTER 1

LITERATURE REVIEW AND RATIONALE

Background

Soybean is a short day annual, belonging in the Leguminosae family. Soybean was introduced into the United States from China in 1765 (Hymowitz and Shurtleff, 2005). It was primarily used as an ornamental plant prior to 1900, and a forage crop from around 1900 through the 1930s. Soybean seed began to be utilized for its high oil content during World War II. The war created a high demand for fats and oils; and cotton seed, which was the primary source to date, was in lower production due to a boll weevil outbreak. Soybean provided a suitable alternative due to its high oil content. Soybean meal also began to be used by producers as a protein source for livestock around this time (McDonald and Copeland, 1997).

United States soybean production nearly tripled from 1940 to 1946, from 2.1 million metric tons (78 million bushels) to 5.5 million metric tons (201 million bushels) (ASA, 2008), and usage continues to expand to date. Producers planted soybeans on 25.7 million hectares (63.6 million acres) in 2007, producing 70.36 million metric tons (2.59 billion bushels) of seed. The average price paid to farmers was $382.00 per metric ton ($10.40 per bushel), which resulted in a greater than $26.8 billion crop (ASA 2008).

Early Phytic Acid Work

United States soybean breeding began prior to 1900, but it was not until the 1930s that state agricultural experiment stations and the USDA initiated breeding programs. High yield, broad adaptation, and to a lesser extent increased seed oil content were early breeding goals, and disease resistance became an important trait in the late 1940s through 1950s (Carter Jr., *et al*., 2004). Whole-plant physiology, plant-water relations, and their impact on yield was a major focus of plant breeding programs in the 1960s and 1970s (Boote and Sinclair, 2006).

In the early 1980s, researchers began to focus on the nutritional attributes of soybean, and work was initiated to decrease the amount of PA in seeds. Raboy, *et al.* (1984) surveyed 163 soybean lines for low PA. The lines contained a range of PA concentrations in their seeds, but none contained low PA levels. Low PA seeds were necessary to further their research, and Raboy and Dickinson (1984) successfully lowered the PA level of soybean seed by fertilizing soybean plants with altered levels of nutrient P. Mature seeds were screened for PA content and then germinated. The nutritionally induced low PA had no effect on seed germination (Raboy *et al*., 1985). While PA is known to provide a substantial store of P for germination and early growth, perhaps its necessity is greater for wild plants than cultivated crops (Raboy, 2001).

Mutation Breeding

Maize

Raboy and Gerbasi (1996) created a population of ethyl methanesulfanate-induced (EMS) maize mutants using a pollen-treatment method (Neuffer and Coe, 1978).

The maize seed used for this study was a population called Early-ACR. Early-ACR plants were pollinated with EMS-treated pollen, producing M_1 seeds heterozygous for the induced mutations. These seeds were planted and self-pollinated to produce the M_2 progenies, which were screened. The first two non-lethal low PA (lpa) mutants found were named *lpa1-1* and *lpa 2-1*. The *lpa1-1* mutant phenotype showed an approximately 66% reduction in seed PA, accompanied by a concomitant increase in available P. The *lpa2-1* mutant phenotype showed an approximately 33-50% reduction in seed PA (Raboy and Gerbasi, 1996; Raboy, *et al*., 2000), with an increase in P_i and two other P-containing compounds, Inositol (Ins) P_4 and Ins P_5. Seed size of the *lpa* mutants decreased from 8-23% in *lpa1-1* and 4-16% in *lpa2-1*. Initial breeding experiments utilized the mutant *lpa1-1* seeds (Ertl, *et al*., 1998). Lpa seeds produced showed "little to no effect" on germination, stalk strength, grain moisture at harvest, or flowering date (Ertl, *et al*., 1998). A 5.5% yield reduction was observed (Raboy, *et al*., 2000).

Barley

Larson *et al*. (1998) treated barley (*Hordeum vulgare* L.) seed with sodium azide, and found multiple mutations, including *low phytic acid* (*lpa1-1* and *lpa2-1*). There was an approximately 45% decrease in PA in *lpa1-1* with a near-equivalent increase in P_i. The *lpa2-1* mutation showed a PA decrease of nearly 70% with increases in P_i and Ins P_5. Bregitzer and Raboy (2006) also conducted experiments on the mutated barley lines, M955, *lpa3-1*, and *lpa1-1* (Dorsch, *et al*., 2003), which contain PA reductions of 95, 65, and 50% respectively, and concomitant increases in available P. The mutant *lpa2-1* (Dorsch, *et al*., 2003) was also studied and found to contain a PA reduction of approximately 40%, with increases in P_i and multiple Ins phosphates.

Field experiments were conducted and no differences were found among mutants for seedling emergence or stand establishment. Mutant plants grown under irrigated conditions appeared as vigorous as the wild-type lines, but under non-irrigated conditions, the mutant plants appeared less vigorous and more susceptible to environmental stress. However, the authors noted that these differences were slight and inconsistent. There was no significant difference in yield for the *lpa1-1*, *lpa2-1*, or *lpa 3-1* mutants and their wild type lines, but M955 did differ significantly in agronomic performance. The *lpa1-1* mutant line was determined to have the greatest potential for development toward an agronomically competitive cultivar due to its superior performance under non-irrigated conditions.

Rice

Gamma-irradiation was used by Larson *et al*. (2000) to mutagenize rice (*Oryza sativa* L.). M2 seeds were screened for the *lpa* phenotype. One non-lethal mutation was identified and named *lpa1-1*. It contained 32% less PA than wild type and a 27% increase in P_i, with little difference in total seed P. Field studies conveying seedling vigor are needed to date.

Soybean

Wilcox *et al*. (2000) created the first lpa soybean mutants by treating the breeding line CX1515-4 with EMS. They crossed this mutant line with the cultivar 'Athow' to develop the line, CX 1834-1-6. The mutation causing the *lpa* phenotype is controlled by recessive alleles at two independent loci, designated *pha1* and *pha2* (Oltmans, *et al*., 2004)

. These alleles exhibit duplicate dominant epistasis, therefore only plants homozygous for the two recessive alleles have the *lpa* phenotype. Seeds grown in the field showed significantly lower emergence rates and plant density than normal PA lines (Hulke *et* al., 2004, Oltmans *et* al., 2005). Meis *et al*. (2003) developed chemically mutagenized soybean lines homozygous for the *mips* allele (Hitz *et al.*, 2002), that both reduced phytate and raffinose saccharide. Seeds were produced in two environments, temperate and subtropical, due to previous results indicating potential climate induced seed vigor issues. Laboratory vigor tests and field emergence tests were conducted. Significant differences among lines and seed sources were found for field emergence, tetrazolium testing, warm germination, cold vigor, and accelerated aging tests in both years of the study. Seeds produced in the temperate climate had a more than three-fold greater field emergence than the subtropical produced seeds.

Wheat

Guttieri, *et al*. (2004) mutagenized wheat (*Triticum aestivum* L.) using EMS, which led to the development of a line containing a 37% reduction in PA. The hexaploid wheat genome may have resulted in a buffering effect which prevented further reduction in PA level. Nevertheless, this line was deemed agronomically unacceptable due to its reduced stature, weak straw, and reduced grain yield. Backcrossing was performed, and subsequent agronomic performance of the reduced PA lines did not differ significantly from lines containing normal levels of PA (Guttieri, *et al*., 2006).

Phytic Acid and Seed Vigor

Breeding for lpa in seeds using mutagenesis has resulted in many questions about the relationship between seed physiology and vigor. Chemical mutagenesis is a broad seed treatment. Chemical mutations are not targeted mutations, and one treatment may result in many genetic changes; most unintended. Raboy (2002) stated that lpa mutations probably affect all plant tissues, not only in the seed. Physiological processes may therefore be altered unintentionally along with lowering the PA level, potentially lowering seed viability and seedling vigor.

In addition to the mutagenesis effects on germination, there are physiological effects of lowered PA (Raboy, 2001). The absence of phytate in seeds alters the chemical composition. Wang et al. (2003) raised concerns regarding micronutrient toxicity in low-phytate seeds, stating that negative consequences for seed viability may occur if micronutrient metals (Fe, Zn, Mn, and Cu) are not properly sequestered in the seed. It is also important to note PA location in the seed. Monocots such as barley, wheat, and rice contain approximately 90% of their PA in the aleurone layer and 10% in the scutellum, and in maize, 90% is found in the scutellum, with 10% in the aleurone layer (as reviewed by Brinch-Pedersen *et al*. 2002). Phytate represents more than 50%, by weight, of the typical small grain aleurone layer (Raboy 1997). In dicots such as soybean, PA is ubiquitous in the cotyledons (Raboy, 2003), and soybean seeds contain approximately 50% more total P than maize, barley, or wheat. Therefore, the reduction in the amount of PA in the *lpa1-1* soybean mutant, for example, is greater than in *lpa1-1* cereal mutants (Wilcox, *et al.*, 2000). Altering seed composition by removing PA may affect monocots and dicots differently. This effect on germination needs further research.

Biotechnology

The decrease in PA achieved through traditional mutation breeding may not be sufficient to achieve adequate nutritional improvements for humans (Raboy, *et al*., 2001). Brinch-Pedersen *et al*. (2002) noted transgenically altering seed for increased phytase activity may prove the most versatile and cost effective solution, but until recently the biotechnology techniques necessary were not developed. Two molecular strategies now utilized to reduce the PA level in seeds involve blocking the PA biosynthetic pathway, and increasing seed phytase activity, which degrades the PA as it is developed (Chiera, *et al*. 2004).

Shi *et al*. (2007) studied the previously mentioned *lpa1* maize mutants to research the molecular basis for undesired agronomic characteristics associated with the mutant lines. They identified an ATP-binding cassette (ABC) transporter that functioned in PA accumulation in seeds. They silenced it in embryos, which resulted in low PA , high P transgenic maize seeds that showed no difference in germination or yield to wild type. Silencing the ABC transporter blocked the PA accumulation while avoiding interference with other metabolic pathways, thus providing a targeted approach and avoiding unintended physiological effects as seen with chemical mutagenesis.

Dassa *et al*. (1990) sequenced the *E. coli* acid phosphatase gene, *appA*, and Golovan, *et al*. (2000) found the *appA* gene to be a bifunctional enzyme, also encoding for phytase activities. This gene was introduced into *Arabidopsis* seed vacuoles using an embryo-specific promoter (Coello, *et al*., 2001). Seeds expressing the *appA* gene showed increased phytase activity, decreased PA, and a concomitant increase in free phosphate,

however no seeds containing low PA or no PA were recovered, leading Coello, *et al*. (2001) to conclude that severe reductions in PA may diminish seed viability. Bilyeu, *et al*. (2008) generated a soybean line (CAPPA) in which an *E. coli* periplasmic phytase, the product of *appA*, was expressed in the cytoplasm of developing soybean cotyledons. The CAPPA line exhibited a high level of phytase expression, ≥90% reduction in seed PA, and a concomitant increase in total free phosphate. The viability and vigor of CAPPA is the focus of this project.

Seed Vigor Testing History

Long before standardized vigor tests, seed exposure to high temperatures prior to testing germination was hypothesized as a potential means to predict seed longevity (Delouche and Baskin, 1973). As early as 1928, U.S. seed technologists began to cite differences in seedling growth by labeling seedlings normal or abnormal in appearance following what were named germination tests (Woodstock, 1973). Franck (1950) modified terminology by suggesting that when tests were conducted under optimum conditions on artificial media, they be called germination tests, but tests conducted in a manner comparable to soil based germination, should be referred to as 'seedling vigor' tests. The use of artificial media based results to predict germination in the field environment continued to raise questions, and Heydecker (1960) proposed his version of the seed germination test and suggested the need for a universal definition of 'seed vigor.' His opinion was that because producers sow seeds in soil, it was inadequate to test germination in/on artificial media, and he proposed germination testing using varied soil conditions. His method was not readily utilized, due to the cumbersome task of comparing soils from different regions in a standardized manner.

By the early 1970s, neither the Association of Official Seed Analysts (AOSA) nor the International Seed Testing Association (ISTA) had developed standards for seed vigor tests (Woodstock, 1973). Abdul-Baki and Anderson (1973) reiterated the need for more accurate vigor testing, and stressed that a quality test required high precision, low cost, and a short duration. Delouche and Baskin (1973) stated the importance of simplicity of any test, its applicability to many plant species, and its reproducibility. Abdul-Baki (1980) gave his definition of seed vigor as a function of rapid, uniform, and high emergence under the broadest range of environmental conditions. Seed vigor tests have since proved themselves an important tool in analyzing the quality of seed lots in relation to field performance (cold test and/or accelerated aging), storability (accelerated aging), and as a measure of physical injury (electrical conductivity and/or tetrazolium) (AOSA, 2002; Woltz and TeKrony, 2001).

Standard Germination Test History

Elias and Copeland (1997) declared the standard germination test the most widely used test of seed vigor. The germination process may be viewed as overlapping and interacting stages: 1) the remobilization of cellular metabolism, which includes increased respiration in the cotyledons during imbibition, providing the necessary ATP production to energize embryonic growth, eventually leading to radicle emergence and 2) the synthesis and maintenance of the enzymes necessary to activate the biochemical pathways leading to cotyledon reserve usage (Ching, 1973). The standard germination test does not account for contamination of samples, abnormality of seedlings, or seedling growth rate (Delouche and Baskin, 1973, AOSA 2002), rather it is a test of viability. A seed is counted as germinated regardless of apparent vigor. The objective of the test is to

maximize germination rates through minimization of seed stress. However, this objective is also the basis for its weakness as a vigor test. Ideal conditions are used, and do not correlate to typical field conditions.

Delouche and Baskin (1973) stated the need for additional testing procedures for accurate seed vigor ranking. Seed quality deterioration is a process, with loss of viability the final outcome of a complex web of vigor loss. Vigor loss begins with a decrease in metabolic activity, including membrane degradation. This causes the seed increased susceptibility to stress, leading to a decrease in the rate of germination, increased susceptibility to pathogens, decreased emergence, and delayed seedling growth. These result in an increase in abnormal seedlings, and all affect the seed prior to the loss of viability. Nevertheless, a combination of seed vigor tests should include the standard germination test, as it is the most accepted test of seed viability (TeKrony and Egli, 1977). The standard germination test is typically the only vigor test conducted on rice (Patin and Gutormson, 2005).

Cold Test History

Corn seed companies popularized the cold test in the 1940s, and by the early 1950s, considerable interest had developed in utilizing a cold test (Rush and Neal, 1951; Goodsell, *et al*. 1955) as a standard vigor testing procedure to obtain a more accurate assessment of seed quality prior to planting than what the standard germination test offered. While the germination testing used to that time provided an quick assessment of viability, the perimeters in which the test were performed were conducive to allowing the maximum number of seeds to germinate under favorable conditions. The problem for

producers was that often the conditions in the field were not favorable, and while the seeds may have germinated under laboratory conditions, the natural spring environment required vigor for which the seed tests may not have adequately screened. Thus, the purpose for developing the cold test was to mimic the early spring field conditions by exposing seed to cold, wet conditions which were favorable for pathogens but not seed germination, followed by warmer conditions in which vigorous seeds would then germinate. Goodsell, *et al*. (1955), modified the protocol by adjusting the moisture level of the seeds prior to testing; this is regarded as the first saturated cold test. Martin, *et al.* (1988), modified the protocol for the saturated test by soaking the seeds in warm water prior to pressing them embryo side down into cold, saturated soil. This additional stress mimics the reduced oxygen environment that may be present in cold, early spring soil (AOSA, 2002).

Despite the advancements of the testing techniques, and the fact that it has been argued that the cold test is the most popular and most widely used vigor test, not including the standard germination test, in North America (Byrum and Copeland, 1995; Woltz, *et al*. 1998), it has yet to be standardized (AOSA, 2002). This is primarily due to varying soil conditions across environments and research locations. Woltz, *et al*. (1998), found results varied due to differences in soil moisture content, cold period temperature, and soil-borne plant pathogens. Other factors such as testing temperature, micro-organism level, and using treated versus untreated seeds were also found to affect the results of the test (Bruggink, *et al.*, 1991). Byrum and Copeland (1995) conducted a study to survey the reproducibility of maize vigor tests by submitting blind samples to various laboratories. They concluded the cold test to be as reproducible as the germination test

under replicated conditions, implicating the advantage standardization would bring. Spear and Fehr (2007) found the cold test to be useful in determining inferior soybean lines, but concluded field evaluation must be included as a means to determine high vigor lines. Until a consensus is made, results will be difficult to replicate. The cold test is widely utilized including crops such as corn (Byrum and Copeland, 1995; Woltz, *et al*., 1998), canola (Elias and Copeland, 1997), soybean (Spear and Fehr, 2007; Byrum and Copeland, 1995; Egli and TeKrony, 1995; Vieira, *et al*., 1999), and rice (Patin and Gutormson, 2005).

Accelerated Aging History

Accelerated aging began to be used as a seed vigor test in the 1970s. The seed industry sought a method to determine the quality of stored seed. It was widely noted that while most commercially sold seed tested high in germination prior to storage, even under identical conditions, seed lot quality was not equal when tested prior to planting. Experiments showed that seed varieties comparable in pre-storage germination were not comparable in their germination rates after storage, and there was no accurate means to predict which seed lots to carryover and which to plant immediately.

Delouche and Baskin (1973) tested the accelerated aging technique on soybean seed as a means to predict high quality soybean varieties. The concept of accelerated aging was already being used in the early 1970s to develop tests to determine the life span of products ranging from insulation on electrical wiring to tent canvas material, and was sought as a potential method to provide a relatively quick assessment of seed vigor. They hypothesized that by exposing soybean seeds to severely adverse levels of the two

most important factors influencing the rate of seed deterioration, high temperature and relative humidity, the deterioration in vigor typically seen from an extended period of storage, would be obtained in a relatively short period of time. The accelerated aging test was standardized following much research regarding techniques (Delouche and Baskin, 1973; McDonald *et al*., 1978; Tao, 1979; Tomes, *et al*., 1988; TeKrony, 2005). Accelerated aging tests have been conducted on many crops, including canola (Elias and Copeland, 1997), maize (Woltz and TeKrony, 2001), soybean (Egli and TeKrony, 1995; Vieira, *et* al., 1999), and rice (Patin and Gutormson, 2005; Yamauchi and Winn, 1996), but the accelerated aging techniques have not been standardized in maize (Woltz and TeKrony, 2001).

Tetrazolium History

The practice of staining seeds for viability evaluation in the U.S. began in 1938 and involved selenium and tellurium salts. U.S. military personnel that had investigated German research brought back information in 1945 regarding the existence and advantages of tetrazolium. Dr. George Lakon and his colleagues in Germany had discovered that the embryonic tissues had to be alive and respiring in order for a seed to germinate. The tetrazolium test for seed vigor was slow to gain acceptance in the United States primarily because the advantages of the results were not yet thoroughly understood by the researchers. Many scientists continued to view seeds as either alive or dead, and the sensitivity of the procedure, the biochemical and physiological implications of the test, and the difficulty of interpreting the results, led many to question its practicality.

It was not until many separate groups around the country began to individually confirm and publish the tetrazolium test's merit that it gained unofficial acceptance by several states in 1961. By this time the question, "How alive are live seeds?", had generated interest, and seed researchers began training each other on the techniques of the tetrazolium test (as reviewed by Moore, 1976; AOSA, 2000). The AOSA officially recognized the tetrazolium test in 1970, and released its first Tetrazolium Testing Handbook, which continues to be the standard protocol. The tetrazolium test is used to estimate both viability and vigor (AOSA, 2002). After a questionable beginning in the seed industry, the tetrazolium test became thoroughly researched and modified to be one of the most rapid and useful methods to test seed viability and vigor (as reviewed by Moore, 1976; AOSA 2002).

Electrical Conductivity History

Imbibition is a requirement for seed germination. Weak or damaged cell membranes cause rapid water uptake and excess electrolyte leakage, which may lead to reduced vigor (as reviewed by Woodstock, 1988). The conductivity test for seed vigor was used to study electrolyte leakage from seeds as early as 1928 by Hibbard and Miller (AOSA, 2002). It became a routine vigor test for garden peas (AOSA, 2002), but did not gain acceptance for use in other crops until the 1980s.

Ching and Schoolcraft (1968) found that increased leaching of sugars from germinating seeds often accompanied a reduction in viability, but Abdul-Baki and Anderson (1970) concluded that this relationship between sugar leaching and loss of seed viability was a complex one, and that seed age and mechanical injury, or the comparison

of accelerated aged seeds and natural aged seeds, resulted in a lack of correlation. Woodstock (1973) summarized the detrimental effects of metabolite leakage from seed tissues and its inverse relationship with vigor into three parts: 1) a loss of membrane integrity, 2) a loss of essential cell constituents, and 3) increased micro-organism activity. Nevertheless, when Woodstock reported this information, he maintained the accepted belief of the time that the electrical conductivity test was essentially restricted to peas due to lack of reproducibility and conflicting research results. Tao (1978) found several factors that affect the results when testing soybean, including differences in filter paper, quality of water, temperature, and seed moisture.

Research on conductivity of soybean seed continued, and acceptance grew. The ability of the electrical conductivity test to measure the leakage of electrolytes provided a different component of the vigor concept than other prominent vigor tests used at the time. Abdul-Baki (1980) proposed the importance of biochemical events that affect vigor maintenance. He related vigor to the condition of the membrane structure and organelle organization during seed imbibition. It was determined that the best correlation of laboratory conductivity testing and field emergence was obtained when the seed sample was random and there was no removal of damaged seeds (Oliveira, *et al*. 1984; Loeffler, *et al*. 1988), leading to increased application of the test as a part of the vigor testing process.

To date research is ongoing to modify the electrical conductivity test for broader application in seed vigor research. It is not recommended that it be the only vigor test utilized, due to concerns that under adverse field conditions, it may not be a reliable indicator of emergence (Vieira *et al*., 1999). Many factors may influence electrical

conductivity results (Pandy, 1992), and single seed analysis remains unreliable (Hamman, *et al*., 2001)**.** When bulk samples are tested, it remains an important component in the vigor testing procedure. The electrical conductivity test has been used on multiple crops, including canola (Elias and Copeland, 1997), rice (Patin and Gutormson, 2005), and soybean (Yaklich, *et al*., 1979).

Field Performance

The ultimate vigor test results from planting a seed into the soil in a field environment, and its subsequent germination, emergence, and establishment. While multiple physiological and biochemical viability and vigor tests provide information on potential seedling vigor, the field environment cannot be replicated in the laboratory. To obtain the most accurate assessment of vigor, laboratory tests should be supplemented with field tests.

Seed germination in the strictest definition incorporates the events that commence with the uptake of water by the quiescent dry seed and terminate with the elongation of the embryonic axis, causing the radicle to penetrate the seed coat (Bewley and Black 1994). Although physiological germination is complete with the elongation of the radicle, seed germination in the field is often considered as full emergence of the cotyledons from the soil (TeKrony and Egli, 1977; Pinthus and Kimel, 1979). Seedling emergence is the result of two distinct phases of growth: germination of the seed and pre-emergent growth (Hamman, *et al*. 2002; Perino and Côme, 1991). Utilizing laboratory vigor testing and field emergence provides supporting vigor information for seeds demonstrating poor seedling emergence.

Hamman *et al*. (2002) asked the question: Is failure to germinate or poor post-germinative growth more problematic to the development of high quality seeds? It is well documented that high seed vigor provides an advantage in stressful environments (Hamman *et al*., 2002; Burris, 1976; Johnson and Wax, 1978; TeKrony, *et al*., 1987). Hamman *et al*. (2002) reported no significant difference in field emergence between low, medium, and high vigor seeds under ideal conditions (sterile soil and shallow planting), however, under stressful conditions (nonsterile soil or deep planting), rapid emergence of seed with greater vigor led to higher stand counts. Low vigor seed may be slower to germinate and the subsequent seedlings slower to emerge, allowing more time for pathogens to infest either seed or seedling. Therefore two seed lots having no difference in a laboratory standard germination tests, may perform differently in the field due to vigor differences (Pinthus and Kimel, 1979; Ferriss and Baker, 1990; TeKrony and Egli, 1991; Hamman, *et al*., 2002).

Vigor experiments in soybean (Egli and TeKrony, 1995; Mason *et al*., 1982; Kulik and Yaklich, 1982; Yaklich, *et al.*, 1979; Yaklich and Kulik, 1979; Johnson and Wax, 1978; TeKrony and Egli, 1977; Edje and Burris, 1971), maize (TeKrony, *et al*. 1989), and rice (Yamauchi and Winn, 1996) have been used to compare laboratory germination to field emergence. Yaklich, *et al*. (1979) found an association between seed leaching and field emergence in soybean, and determined the conductivity of the seed leachate could be used as a vigor test correlated to field emergence. The standard germination test, cold test, accelerated aging, and tetrazolium tests all positively correlated to field emergence of soybean in experiments conducted by Kulik and Yaklich (1979, 1982).

Field conditions such as the temperature and structure of the soil, moisture, and microorganisms all affect the ability of a seed to germinate and emerge (Burris, 1976). Egli and TeKrony (1995) summarized this variation in emergence by stating that the relationship between seed quality and field emergence is dependent on seedbed conditions, and the subsequent results of vigor tests were reliant on these conditions.

After emergence and equal stand establishment, multiple groups studied the relationship between seed vigor and yield and found no significant difference (Kulik and Yaklich, 1982, TeKrony *et al*. 1987, Egli and TeKrony, 1979, Edje and Burris, 1971), leading to the conclusion that seed vigor does not influence yield, although it may be argued that seed vigor does in fact affect yield, because a cultivar with lower vigor would produce a poorer stand count (as reviewed by TeKrony and Egli, 1991).

It is well known that generally plants can compensate for their environment. For example, in lower plant density environments, soybean plants branch more profusely (Heatherly and Elmore, 2004), leading to increased flower production, pod set, and subsequent seeds per plant. Egli (1988) found increased yield with higher plant densities, but Kakuichi and Kobata (2004) found an increase in seeds per plant resulted from lower plant densities. Vega, *et al*. (2001) studied seed number per plant as it related to vegetative growth. They found a linear relationship between number of seeds per soybean plant and plant growth rate, which they attributed to its reproductive plasticity. Kakiuch and Kobata (2004) thinned rows of soybean plants to study harvest index, and found reduced plant density resulted in increased seeds per plant and pods per plant.

Norworthy and Frederick (2002) studied seeding rate for glyphosate-resistant soybean varieties, and found that lower seeding rates resulted in lower main branch seed yield, compared to plants in the recommended seeding rate plots. However, while reduced seeding resulted in 40% fewer plants, the seed yield of these plants did not differ significantly from the yield of stands at the recommended seeding rate due to more soybean branching and higher seed production on those branches. Troesser (2008) found soybean compensated for lower plant density in the field, and interplanting of additional seed proved useful, however replanting of seed when the stand count was sparse wasn't economical.

Crop improvement requires knowledge of growth and physiology during the entire life of the plant. The analysis of seeds per fruit, fruits per plant, and plants per area studied are all necessary to obtain accurate information on growth and development. While final yield numbers indicate productivity, an integrated study approach to the components of yield is useful.

Growth analysis is the quantification of yield-influencing environmental effects on plant development (Gardner, *et al*., 1985). Koller, *et al*. (1970), defined growth analysis as the quantification of the components of crop growth, and they were among the first to analyze the growth of soybean. They found a correlation between assimilate demand (sink) and photosynthetic activity.

McGraw and Beuselinck (1983) conducted a growth analysis of the legume, birdsfoot trefoil (*Lotus corniculatus* L.) to study yield impediments resulting in seed shatter and uneven pod maturity. Vegetative and reproductive plant tissues were

separated, followed by further separation of the reproductive tissues into seeds and pods which allowed for quantification of growth and development over time. Interplant competition also affects yield, and may prevent an accurate assessment of potential vigor and subsequent yield.

Fasoula and Fasoula (2002) defined competition as the plant-to-plant interference in the sharing of growth resources caused by genetic and environmental differences. They developed a seed planting system named honeycomb, in which seeds were field-planted in a uniformly spaced manner, minimizing interplant competition and maximizing the potential for individual plant growth. Fasoula and Boerma (2004) used this planting system to study protein, oil, and fatty acid content of soybean, and later seed weight, plant height and maturity, lodging, and seed yield (Fasoula and Boerma, 2007).

It may be accepted that thorough testing of seed vigor is essential to development of quality varieties. Soybean research continues to exemplify the collaboration between the laboratory and the field, as many researchers utilize a combination of seed vigor tests and field emergence to gain a thorough representation of different aspects of seed vigor and field emergence (TeKrony and Egli, 1977; Johnson and Wax, 1978; Yaklich and Kulik, 1979; Yaklich, *et al*., 1979; Kulik and Yaklich, 1982; Mason, *et al*., 1982; Ferriss and Baker, 1990; Vieira, *et al*., 1992; Egli and TeKrony, 1995; Hamman *et al*., 2002).

Rationale

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate; PA) provides seeds and developing seedlings a stored supply of phosphorus (P) and cations necessary for germination and early growth. It is also widely considered the most important anti-

nutritional factor for the availability of minerals such as zinc, calcium, and iron in the food and feed of humans and monogastric animals (as reviewed by Brinch-Pedersen 2002). PA is synthesized in immature seeds beginning approximately 14 days after the onset of anthesis, which is the R1 stage (Fehr, *et al*. 1971) in soybean (*Glycine max*, [L.] Merr.), and increases approximately linearly from 21 days after anthesis through maturity (R8) (Raboy and Dickinson, 1987). Total P in a seed ranges from 3.0 to 4.0 mg g^{-1}. PA typically represents from 65% to 80% (2.0 to 3.0 mg g^{-1}) of this, which is one to several percent of the total seed weight. It is primarily found in the form of phytate or phytin in seed (Raboy 1997). Phytate is a salt, formed when phytic acid chelates a mix of divalent cations, including Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, and Cu^{2+}. Phytin is the salt formed when phytic acid chelates only one type of the divalent cations.

PA chelates essential nutrients, and is considered undesirable, because monogastric animals lack the enzyme phytase. When monogastrics consume feed derived from seeds, they are unable to utilize the phosphorus and cations from phytate. Undigested phytate is lost through excretion, causing a nutritional concern. There would be sufficient P present in corn (*Zea mays*) and soybean meal to meet the P requirements of monogastric livestock if it were available (Cromwell and Coffey, 1991). Spencer, *et al*. (2000) found P in low-phytate feed to be approximately five times more available to swine than that in normal-phytate corn. Digestibility also increased, leading to a significant reduction in the amount of phosphorus excreted in the waste. They also found an increase in calcium utilization in animals consuming low-phytate feed, and suggested the possibility that the digestibilities of other cations or amino acids may also be enhanced by the use of low-phytate material.

Undigested phytate is also a potential environmental concern. It adsorbs soil particles, and poor conservation practices may result in soil erosion, which may lead to runoff into bodies of water. While conservation practices to reduce soil erosion diminish this concern, reducing the excretion of P by livestock is also an important variable in the environmental aspect of this issue (Cromwell and Coffey, 1991; Sharpley, *et al*., 1994; Ertl, *et al*., 1998).

Producers use two approaches to mitigate the P problem. One approach is to supplement livestock feed with a fungal derived phytase (Cromwell and Coffey, 1991; Ertl *et al*., 1998; Spencer *et al*., 2000). Cromwell, *et al*. (1993) increased the bioavailability of P from 25% in a soybean meal diet to 57% by supplementing with phytase. In a corn-soybean diet, phytase supplementation increased the available P from 15 to 43% (Cromwell, *et al*., 1993). Jongbloed, *et al*. (1992) found that supplementing feed with phytase from *Aspergillus niger* significantly reduced the concentration of total P in the feces of pigs. The addition of inorganic phosphates such as dicalcium phosphate to the feed is another common approach (Cromwell and Coffey, 1991), but does not reduce the undesired phytate in the feed, resulting in a higher level of undigested P excreted in the waste. Cromwell and Coffey (1991) reported that a 50% reduction in excreted P would result in 15 million tons less P entering the U.S. environment annually.

A third approach to mitigate the PA problem is through genetic manipulation. Chemical mutagenesis has been utilized by plant breeders (Larson, *et al*., 1998; Raboy, *et al*., 2000; Wilcox, *et al*., 2000; Larson, *et al*., 2000; Guttieri, *et al*., 2004) as a means to lower phytic acid in seeds. However, unintended mutational conformations caused by the chemical mutagenesis may affect physiological processes, namely seed germination and

seedling vigor (Raboy, 2002). Biotechnology provides transgenic approaches and molecular biology techniques to potentially mitigate the unintended physiological effects that may be encountered in mutated germplasm.

The soybean cultivar, 'Jack' was transformed through insertion of an *Escherichia coli* periplasmic phytase, the product of the *appA* gene, expressed in the cytoplasm of developing cotyledons (Bilyeu, *et al*., 2008). The transformed line, CAPPA, has high phytase activity, >90% reduction of PA, and a concomitant increase in available P without any negative conformational changes.

Using transgenic methods to combat the vigor concerns of conventionally mutated low phytate seeds has great promise, but seed vigor testing is an essential part of the process prior to public release of seeds. My project involves testing the viability, vigor, and yield of CAPPA in the laboratory and field. While many researchers have studied soybean seed vigor and its relationship to field emergence (Egli and TeKrony, 1995; Kulik and Yaklich, 1982; Yaklich, *et al*., 1979; Yaklich and Kulik, 1979; Johnson and Wax, 1978; TeKrony and Egli, 1977; Edje and Burris, 1971), this research is the first to study a transgenic soybean line high in phytase activity with undetectable levels of PA for its viability, vigor, and field performance.

Current public perception of using transgenic seeds for food and feed is mixed, and the use of transgenic plants for human consumption remains a debated issue. There is a lack of public knowledge regarding this area, and while acceptance would be beneficial to research involving transgenic seeds, dissemination of research based facts may be necessary to achieve this. As the public becomes more informed, it is anticipated that

consumers will respond positively to transgenic products that contain improved nutritional quality (Byrne, 2006).

Objectives

The objectives of this project were to achieve a better understanding of the consequences of expression of high levels of phytase in developing CAPPA soybean seeds in terms of viability, vigor, and field performance. Previous research failed to define the relationship between seed composition changes and subsequent viability and vigor of the modified seeds. My first hypothesis was that there would be no significant difference between CAPPA and its progenitor, Jack, in the field environment. To test this, I conducted two studies to evaluate the emergence and dry mass gain of plants grown in the field. My second hypothesis was that CAPPA vigor would not be significantly lower than Jack because the transformation was a targeted approach, i.e. there would be no unintended physiological effects in CAPPA, as seen with chemically mutagenized soybean lines. To test this I conducted five laboratory vigor evaluations. The results indicate CAPPA vigor appeared to be lower than Jack; field emergence results were not conclusive. Emerged CAPPA plants grew to be competitive, high yielding plants.

CHAPTER 2

MATERIALS AND METHODS

Field Studies

Four soybean entries, Williams 82, Jack, CAPPA, and CX1834-1-3, were grown at the Bradford Research and Extension Center (38° 53' 40" N, 92° 12' 20" W) in 2006 and 2007. The soil at this location was a Mexico silt loam, 1 to 3 percent slope, eroded (Fine, smectitic, mesic Aeric Vertic Epiaqualf). Soil sampling was conducted in the spring of each year prior to planting using MU Extension Soil Testing Laboratory guidelines. Twenty, 17 cm soil cores were extracted uniformly from the field, mixed together, and a subsample of the mixture was submitted for testing (Table 9). The field was disked and cultivated using standard agricultural methods, and split into two study plots.

Two studies were conducted each year: a seedling dry mass study (SDMS) and a yield component study (YCS). Experimental design for each study was a randomized complete block (RCB) with four replications and rows on 0.8 m centers. SDMS seeds were hand planted into rows at a depth of 3 cm every 30 cm to maximize stand count accuracy and minimize seedling competition and spread of disease. YCS seeds were inoculated with *Bradyrhizobium japonicum* and hand-planted at a depth of 3 cm. Seedlings were later thinned to obtain one plant every 0.8 meters to minimize interplant competition. Pre-emergent herbicide was applied in 2006 and 2007 (Table 10). Windy conditions prevented timely application of the pre-emergent herbicide in 2007. Border

row seedlings began to emerge prior to application of the pre-emergent herbicide, resulting in the need to change herbicides.

Emerged seedlings, those with cotyledons free from soil (TeKrony and Egli, 1977), were flagged at seven days after planting (7 DAP) in the SDMS. Two seedlings per experimental line per replication per day for each of 14 days (7-21 DAP) were extracted from the soil, stored on ice, and transplanted to the laboratory. Seedlings were washed of soil, separated into vegetative components (hypocotyl and stem, cotyledons, and leaves), frozen in liquid N, and then stored at -80° C. The separated vegetative samples were lyophilized then weighed. A final emergence count was made 14 DAP (TeKrony and Egli, 1977). Statistical analyses of seedling dry mass differences over the sampling period were conducted using PROC ANOVA (SAS Institute, 2003). Differences between mean dry mass of each entry over the sampling period and differences between each line's dry mass on individual sampling dates were analyzed.

Sampling for the YCS began at the R5 stage. One plant per experimental line per replication per week (through R8 stage, 6-7 wks) was sampled at soil level, stored in a sealed bag and refrigerated (4°C) until analysis in the laboratory. Plants were separated into their vegetative and reproductive parts. Pods were kept intact. Vegetative components (leaves, branches, and stems) were oven dried and individual components weighed. Intact pods were frozen (-20°C), lyophilized, and weighed. YCS R5-R7 data were subjected to statistical analyses using PROC GLM (SAS Institute, 2003) to test year and entry effects for total vegetative weight (stems, branches, and leaves combined), total reproductive weight (combined pods and seeds), and total weight (vegetative and reproductive components combined).

Sampling at R8 in the YCS did not include sampling vegetative components. R8 plants were single plant threshed and seeds were stored at 4º C. Total numbers of seed per plant were recorded, outliers were sieved and discarded using size 19 and 13 round hole sieves, and 100 seed weights were determined. Lots of one hundred seed from each plant were subjected to standard germination testing (AOSA, 2007) and accelerated aging testing (AOSA, 2002). Forty seeds from each plant were ground in a coffee grinder (Black & Decker SmartGrind CBG5, Towson, MD) and subjected to fatty acid analysis, total N, total protein, available P, phytase activity including the Bradford assay, PA, and macronutrient/ micronutrient content. YCS data were subjected to analyses using PROC ANOVA (SAS Institute, 2003) to test for differences between years' entries, and year by entry interaction.

Available P Assay

Available P was quantified by the method described by Bilyeu *et al*. (2008). Ground seed samples (0.011-0.014 g) were extracted overnight by shaking at 4ºC in 0.5 mL of extraction buffer [12.5% (w/v) TCA, 25 mM $MgCl_2$]. Particulates were allowed to settle at room temperature for 30 min to 1h. Ten microliters of sample supernatant were mixed with 90 µL water and 100µL colorimetric reagent [1 volume of 3 M H_2SO_4, 1 volume of 0.02 N ammonium molybdate, 1 volume of 10% (v/v) ascorbic acid, and 2 volumes of water] in a 96-well plate well, allowed to incubate at room temperature 1.5 h, and then analyzed at 825 nm in a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA). A standard curve was generated with K_2HPO_4 standards. Results were converted to µg P mg $seed^{-1}$. Statistical analysis was conducted for available

P differences between years, entries, and year by entry interaction using PROC ANOVA (SAS Institute, 2003).

PA Assay

PA was quantified by the HPLC method described by Bilyeu *et al*. (2008). Ground seed samples (0.025 g) were extracted 1 h by shaking at room temperature in 0.5 mL of 0.5 N HCl. After centrifugation, 15 min at 15,000*g*, supernatants were filtered through a 0.22-micron filter, and 100 µL of filtrate was analyzed. PA and inositol polyphosphate separations were performed by a linear gradient elution program on a Dionex CarboPac^{TM} PA-100 guard column (Dionex, Sunnyvale, CA) and a CarboPac^{TM} PA-100 analytical column (Dionex, Sunnyvale, CA) on an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA).

The elution gradient was effected by a mixture of two eluents: water and 0.5 N HCl; time 0 min, 8% 0.5 N HCl; time 30, 100% 0.5 N HCl; time 35, 100% 0.5 N HCl; time 35.1, 100% 0.5 N HCl; time 40, 8% 0.5 N HCl. A post-column derivitization was achieved with a solution of 1 g L^{-1} $Fe(NO_3)_3$ in 0.33 M $HClO_4$ using a 750-µL knitted coil and was followed by detection of absorbance at 295 nm. Flow rates of eluent and post-column solution were 1.0 and 0.4 mL min^{-1}, respectively. PA standard (phytic acid dipotassium salt; Sigma-Aldrich, St. Louis, MO) was eluted at 30 min, while a myoinositol-1,3,4,5,6-penta*kis*phosphate (Sigma-Aldrich, St. Louis, MO) standard eluted at 23 min. Standard curves were calculated from dilutions of PA standards. Results were converted to µg PA mg $seed^{-1}$ or PA-P mg $seed^{-1}$. Statistical analysis was conducted for

PA differences between years, entries, and year by entry interaction using PROC ANOVA (SAS Institute, 2003).

Phytase Assay

Phytase was quantified by minor modifications to the method described by Bilyeu *et al*. (2008). Ground seed samples (0.025 g) were extracted in 1 mL of enzyme buffer (0.1 M sodium acetate, pH 5.5, 1 mM $CaCl_2$, and 0.1 g L^{-1} Tween 20) by thorough vortex mixing, followed by incubation on ice 10 min. Samples were centrifuged 5 min at 15,000*g*, 4ºC. The supernatants were then either diluted 20 fold in enzyme buffer or sampled without further dilution. This differs from Bilyeu, *et al*. (2008), as they diluted supernatants 50 fold in enzyme buffer or sampled without further dilution. With solutions cooled on ice, a 40-µL aliquot of sample was combined with 680µL of reaction buffer (0.1 M sodium acetate, pH 4.5) and 80 µL of 12.5 mM PA. For the zero time point, 125 µL of the mixture was removed and mixed with 125 µL of cold 20% TCA, vortexed, and placed on ice. The remaining reactions were incubated 15 min in a 37ºC water bath. Reactions (125 µL) were stopped as for the zero time point. All samples were centrifuged 5 min at 15,000*g*, 4ºC.

Available P content was quantified as above with 100 µL of sample combined with 100 µL of colorimetric reagent, except that standards were assayed in 10% TCA rather than water. Extracted protein was quantified against bovine serum albumin standard according to the recommendations of the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Results were converted to phytase specific activity: µmol Pi released·min^{-1}·mg $protein^{-1}$. Statistical analysis was conducted for phytase

activity differences between years, entries, and year by entry interaction using PROC ANOVA (SAS Institute, 2003).

Macro and Micronutrient Analysis

Ground seed (0.5 g) was ashed 5 h at 500ºC and dissolved in 10 mL of 6 N HCl. Digested samples were diluted with water, filtered, and analyzed for N, P, K, Ca, Mg, Zn, Fe, Mn, Cu, and B by ICP-OES (Varian, Inc., Walnut Creek, CA). Nitrogen content of ground seed was quantified by thermal conductivity of nitrogenous gases with a LECO TruSpec model FP-428 nitrogen analyzer (LECO, St. Joseph, MI), then converted to percent protein content based on a protein factor of 6.25. Statistical analysis was conducted for macronutrient and micronutrient differences between years, entries, and year by entry interaction using PROC ANOVA (SAS Institute, 2003).

Fatty Acid Analysis

Fatty acid analysis was determined by the method used by Bilyeu, *et al*. (2005) with minor modifications. The concentration of each fatty acid in the sample was determined as a percentage of the total fatty acids of the seed by lipid gas chromatography of fatty acid methyl esters of extracted oil. Ground samples (0.067-0.073g) were extracted overnight in 1 mL of a chloroform-hexane-methanol (8:5:2, v/v/v) extraction buffer at room temperature. This differs from Bilyeu, *et al*. (2005), as they extracted the oil from seed chips, not whole ground seed samples. Derivatization of 150 mL of solvent was done with 75 mL of methylating reagent (0.5 *M* methanolic sodium methoxide-petroleum ether-ethyl ether, 1:5:2, v/v/v). Samples were diluted with hexane to 1 mL. An Agilent (Palo Alto, CA) series 6890N capillary gas chromatograph fitted

with a flame ionization detector (2758C) was used with an AT-Silar capillary column (Alltech Associates, Deerfield, IL). Statistical analysis was conducted for fatty acid differences between years, entries, and year by entry interaction using PROC ANOVA (SAS Institute, 2003).

Standard Germination

Standard germination testing was performed on 2006 and 2007 seed samples from the YCS according to AOSA (2007) protocol. Four replications of 50 seeds were tested for each cultivar. Seeds were placed between moistened folded paper towels in open Ziploc bags (S.C. Johnson & Son, Inc., Racine, WI), and stored at 25°C in a horizontal position. Germination counts were made at 5 and 8 days. Seeds were counted as germinated when hypocotyls were ≥2 mm. Statistical analysis was conducted for differences between years, entries, and year by entry interaction using PROC ANOVA (SAS Institute, 2003).

Accelerated Aging

Accelerated aging testing was performed on 2006 and 2007 seed samples from the YCS according to AOSA (2002) protocol. Four replications of 50 seeds were tested for each entry. Seeds were weighed and placed single layer on a screen tray in a seed aging box containing 40 mL ultra pure, deionized water. The AOSA protocol requires 42 g of seed for proper testing. Additional seeds of commercial soybean were added to the tray as needed to meet the weight requirement. The treatment entry seed were separated from the commercial seed by a plastic divider. The closed aging boxes were incubated in the dark at 41°C for 72 hours. Following the treatment for accelerated aging, seeds were

transferred to moistened folded paper towels as previously described and standard germination tests conducted. Statistical analysis was conducted for differences between years, entries, and year by entry interaction using PROC ANOVA (SAS Institute, 2003).

Laboratory Vigor Experiments

Seed of the four soybean entries, Williams 82, Jack, CAPPA, and CX1834-1-3, were produced at the Bradford Research and Extension Center (38° 53' 40" N, 92° 12' 20" W) as previously described in 2006 for utilization in laboratory vigor experiments. Seeds were harvested at plant maturity (R8) and each entry was stored separately at ~21ºC until further analysis.

Electrical Conductivity

Four replications of 50 seeds of each entry were tested for electrical conductivity according to AOSA (2002) protocol. Seeds were placed in 125 mL Erlenmeyer flasks containing 75 mL ultra pure deionized water equilibrated to 25°C, then maintained at 25°C for 24 h. After 24 h of soaking, the flasks were swirled for 10-15 sec, and the conductivity dip cell was inserted into the seep water until a stabilized reading was achieved. The dip cell was not allowed to touch the sample seeds. The conductivity dip cell was rinsed once in each of two beakers of rinse water between each sample measurement. After all samples within a replication were tested, the KCl solution was recalibrated with the electrical conductivity meter, and the rinse water was replaced and conductivity tested prior to testing the next replication.

The conductivity meter (Mettler-Toledo, Columbus, OH) was calibrated using a 0.01 M KCl solution. Enzyme grade KCl (Sigma-Aldrich, Corp., St. Louis, MO) was

dried 2 h at 75°C then cooled in a desiccator prior to weighing. AOSA (2002) protocol required the 0.01 M KCl solution to be 1,408 $\mu S\ cm^{-1} \pm 1\text{-}5\ \mu S\ cm^{-1}$ at 25ºC; the prepared 0.01 M KCl testing solution calibration was 1,414 $\mu S\ cm^{-1}$. The conductivity of ultra pure, deionized dip cell rinse water was also tested prior to testing the samples, and was 0.47 $\mu S\ cm^{-1}$, which was below the 2.00 $\mu mhos\ cm^{-1}$ maximum conductivity for rinse water. Statistical analysis was conducted for differences between replications and entries using PROC ANOVA (SAS Institute, 2003).

Tetrazolium

Four replications of 50 seeds of each entry from 2006 field grown material were tetrazolium tested (AOSA, 2000). Seeds were pre-conditioned for staining by placing seeds between paper towels moistened with ultra pure, deionized water for 18h at 25ºC. Following preconditioning, seeds were treated in a 1% tetrazolium solution [0.067 M potassium phosphate (KH_2PO_4), 0.067 M sodium phosphate (Na_2HPO_4), 2:3, v/v; 10g of 2,3,5-triphenyl tetrazolium chloride (TTC)] at 35°C for four hours. Treated seeds were dissected, and the extent of tetrazolium solution uptake by infiltration of the tetrazolium into the cotyledons was recorded, where viable tissue stained pink, and non-viable tissue was unstained. Statistical analysis was conducted for differences in viability between replications and entries using PROC ANOVA (SAS Institute, 2003).

Cold Test

Four replications of 50 seeds of each entry from 2006 field grown material were cold tested by the method described by Vieira, *et al*. (1992) with minor modifications. Kimpak germination paper (Seedburo Equipment Company, Des Plaines, IL) was placed

in a tray, moistened with 500 mL ultra pure, deionized water and placed in 10ºC growth chambers for 24 hr. Soil from the A horizon was collected from near the research plot used for field studies, spread in trays, and placed in 10ºC growth chambers for 24 hr. Seeds were scattered over the chilled Kimpak at the rate of 50 seeds (one replication of one line) per tray, and chilled soil was spread over the seeds. Trays of soil covered seed were treated at 10ºC for seven days. Ultra pure, deionized water (200 mL) was added to the trays on 6 DAP. At 7 DAP, growth chamber temperature was adjusted to 25ºC for four days. Ultra pure, deionized water (100 mL) was added to the trays on 9 DAP. Emerged seedlings, those with cotyledons free from soil (TeKrony and Egli, 1977), were counted on 11 DAP. Statistical analysis was conducted for differences between replications and entries using PROC ANOVA (SAS Institute, 2003).

CHAPTER 3

RESULTS AND DISCUSSION

Seedling Dry Mass Study (SDMS)

Field emergence was not significantly different between the four soybean entries in this study in 2006 (Table 1), but is noted to have been poor. Emerged seedlings with broken hypocotyls were noted as emergence counts were made, indicating adequate germination followed by impediments to emergence.

Factors contributing to the poor emergence included rain and soil crusting. This study was planted 8 June, and heavy precipitation (6.1 cm) on 10 and 11 June was followed by clear days with temperatures between 30-33ºC during 15-21 June. Subsequent soil crusting may have impeded seedling emergence. Irrigation (1.3 cm) was applied on 20 June to aid in loosening the top soil, but was not successful in improving seedling emergence. TeKrony and Egli (1977) found adverse soil conditions, including soil crusting, not only lowered soybean seedling emergence, but caused substantial variation in emergence among entries. Hyatt, *et al*. (2007) found a significant decrease in soybean seedling emergence and an increase in the number of seeds that germinated but did not emerge as soil compaction increased. They found high vigor seed lots to be significantly higher in emergence, but all lots showed reduced emergence with increased compaction.

In 2007, environmental stress was considered to be less than in 2006, and may have contributed to improved seedling emergence, relative to 2006. Differences in

seedling emergence among the entries were observed. Williams 82 and Jack did not differ in emergence, and were higher than CAPPA and CX1834-1-3. CAPPA emergence was significantly lower than the other three entries in 2007, demonstrating that CAPPA exhibited reduced vigor.

Hulke, *et al*. (2004) and Oltmans, *et al*. (2005) reported that CX1834 derived lines consistently showed a reduced emergence relative to 'normal phytate' lines. The field emergence results from 2006 and 2007 demonstrated CX1834-1-3 emergence did not differ significantly from Williams 82 or Jack. CAPPA emergence did not differ from the other three entries in 2006, but was lower than the other three entries in 2007. The year-to-year variability of the field environment indicates further testing is needed to confirm these observations.

Poor emergence of the four entries in 2006, as previously shown, precluded the evaluation for seedling dry mass in that year. Cumulative above ground seedling dry mass for the four soybean entries in 2007 are presented in Figure 1. Williams 82 seedlings produced significantly greater mean above ground dry mass than the other three entries. There was no difference between Jack, CAPPA, and CX1834-1-3 for mean seedling dry mass averaged over the sampling period. Generally, the dates of sampling, starting at 8 DAP until 21 DAP, revealed considerable sampling variation among the entries and dates of sampling. The differences among the four entries was often not significant on a specific sampling date, especially between 8 and 14 DAP. At almost every sampling, seedlings of Williams 82 produced the most dry mass, while seedlings of CAPPA exhibited the lowest dry mass. Entries began to exhibit more consistent differences in growth and dry mass accumulation beginning 17 DAP (Figure 1), but the

seedling dry mass of CAPPA and Jack were not significantly different on any sampling date in this portion of the study, indicating that CAPPA was equal to its parent line, Jack; i.e. the transformation of Jack for the production of high phytase does not appear to be detrimental to post-germination seedling growth. This finding supports Egli, *et al*. (1990), who reported that seed vigor did not affect the relative growth rate of 'normal' soybean seedlings. CX1834-1-3 seedling dry mass increased 0.529 g between 20 and 21 DAP, and the other entries averaged 0.244 g dry mass increase during that period (Figure 1). CX1834-1-3 averaged 0.040 g dry mass increase per day between 8 DAP and 20 DAP, so the increase in growth rate was noted, but because sampling concluded 21 DAP, it remained unanswered whether this increase was an artifact of sampling due to small sample size, or a true indicator of CX1834-1-3's growth rate. Exponential growth analyses to predict continuums in the growth rate of the four entries through 28 DAP are presented in Figure 2. The growth curves predict that Williams 82 plants would be heavier than the other three entries out to 28 DAP, and CX1834-1-3 plants may perform more closely to CAPPA than Williams 82 or Jack.

Yield Component Study Plant Analysis

Total above ground dry mass of the four entries was sampled weekly in 2006 and 2007 commencing at R5 (72 and 70 DAP, respectively) and concluding at R7 (107 and 98 DAP, respectively). In 2006, Jack and Williams 82 plants were significantly heavier than CAPPA and CX1834-1-3 averaged over the sampling period. On the first sampling date (72 DAP) in 2006 (Figure 3), Jack and Williams 82 were not different, but CX1834-1-3 and CAPPA produced significantly less dry mass than Jack. When those plants were separated into their vegetative and reproductive components, the total vegetative weight

of Jack was greater than the other three entries (Figure 4), but there were no differences in reproductive weight between the four entries at 72 DAP (Figure 5). At 79 DAP, Jack was significantly heavier than the other three entries (Figure 3), and the vegetative weights of the entries mirrored their total weight (Figure 4) although there were no differences among the entries for reproductive weight (Figure 5). The total weight of Jack was again greater than the other entries at 86 DAP (Figure 3), and was observed in the vegetative and reproductive weights of Jack (Figures 4 and 5). Pod filling was evident by reproductive differences by 86 DAP (Figure 5). Williams 82 plants at 86 DAP exhibited delayed pod filling and yielded the lowest sample weight (Figure 5), but not significantly different from CAPPA. At 93 DAP, Williams 82 and Jack did not differ in total weight (Figure 3) or vegetative weight (Figure 4), but were significantly heavier than CX1834-1-3 and CAPPA. CAPPA exhibited delayed maturity at 93 DAP, and its pods and seeds were significantly lighter than the other entries (Figure 5). Although plants of Jack produced consistently greater dry mass than CAPPA by an average of 40% during the 28-day sampling period, by 100 DAP Jack and CAPPA reproductive weights did not differ. Reproductive samples were incompletely lyophilized, and the residual moisture promoted their spoilage. The spoiled samples were from multiple sample days in 2006, but were extensive from the R7 (107 DAP) stage. Therefore only vegetative components from the R7 (107 DAP) stage in 2006 were analyzed; i.e. weights of reproductive portions and total plants from the 107 DAP sampling are not reported.

Entry sample plant weights were two times greater in 2007 (Figure 6) than 2006 (Figure 3). The reproductive sampling period in 2007 was one week shorter than in 2006 due to accelerated plant maturity. Jack and CAPPA plants did not differ in total weight

(Figure 6) averaged over the sampling period in 2007, but the vegetative components of plants of Jack were heavier than CAPPA (Figure 7), and the reproductive mass of CAPPA was greater than Jack (Figure 8). Jack and CAPPA total weights (Figure 6), vegetative weights (Figure 7), and reproductive weights (Figure 8) at 70 DAP were not different (P=0.05). Williams 82 plants were heavier than the other entries at 77 DAP (Figure 6), but CAPPA reproductive weight was greater than the other entries, which did not differ (Figure 8). CAPPA, Jack, and Williams 82 did not differ in total weight at 84 DAP (Figure 6), and were heavier plants than CX1834-1-3. At 84 DAP Williams 82 and Jack vegetative weights were greater than CAPPA and CAPPA vegetative components were heavier than CX1834-1-3 (Figure 7). CAPPA, Jack, and CX1834-1-3 reproductive components did not differ at 84 DAP, but were heavier than Williams 82 (Figure 8). The total weights of the four entries at 91 DAP did not differ (Figure 6), but the vegetative components of Williams 82 were heavier than those of the other entries (Figure 7), and CX1834-1-3 reproductive components were heavier than those components of the other entries (Figure 8). Williams 82 and Jack did not differ in vegetative weight at 98 DAP (Figure 7) and were larger plants than CAPPA and CX1834-1-3, but CAPPA and CX1834-1-3 reproductive components were significantly heavier than those components of Jack and Williams 82 at the R7 (98 DAP) stage (Figure 8). By the R7 stage, there were no differences between the entries for total above ground dry mass (Figure 6). Williams 82 and Jack vegetative components had greater dry mass than those of CAPPA and CX1834-1-3 (Figure 7), but CAPPA and CX1834-1-3 reproductive dry mass was greater than those of Williams 82 and Jack (Figure 8). These data demonstrate that CAPPA did not differ from its parent line, Jack; i.e. the transformation of CAPPA for high phytase

did not affect reproductive component mass. Reproductive samples were again inadvertently destroyed by inadequate lyophilizing in 2007, but there were no sampling days in which all reproductive samples were destroyed, allowing analysis for each sample day to be possible.

Yield Component Study R8 Seed Analyses

Yield Components

Mean number of seeds per plant, seed weight, and yield of R8 seeds of the four entries from the 2006 and 2007 Yield Component Study are presented in Table 2. Entry seeds weighed significantly more in 2006 than 2007, but plants grown in 2007 produced significantly more seeds per plant, resulting in significantly greater yield in 2007.

Among the entries, CAPPA produced the greatest mean number of seeds per plant in 2006, and considerably more than CX1834-1-3, the other low phytic acid entry. However, in 2007 the number of seeds per plant produced was not different among the four entries. CX1834-1-3 seeds weighed significantly more than the other entries in 2006, but in 2007 CX1834-1-3 and Williams 82 seed weights were not significantly different, and CAPPA and Jack seed weights were significantly lower than CX1834-1-3 and Williams 82. Although CX1834-1-3 exhibited the lowest mean seed yield among the entries in 2006, it reversed performance in 2007, as seed yield was determined by number and weight of seeds; i.e., CX1834-1-3 produced heavy seeds, but the number of seeds per plant account for the yield differences between 2006 and 2007. In contrast, the yield of CAPPA was relatively stable over the same period and environment. Yield was calculated based on equal number of plants from each entry, and there was no difference

in Jack and CAPPA, as expected. Egli and TeKrony (1979) found no yield advantage with high vigor seed when stand counts were equal, which agrees with this data.

Phosphorus, Phytic Acid, and Phytase Activity

Seed available P, PA, and phytase activity of R8 seeds of the entries from the 2006 and 2007 Yield Component Study are presented in Table 3. For all the entries, available seed P was greater in 2006 than 2007, but seed PA and phytase activity did not differ. Seeds of CAPPA contained significantly greater available P, undetectable levels of PA, and significantly greater phytase activity than the other entries. This was expected, and consistent with preliminary experiments (data not shown). Seeds of CX1834-1-3, the other low phytate entry, also contained more available P than seeds of Williams 82 and Jack, but not as much as seeds of CAPPA. Seeds of CX1834-1-3 also exhibited increased phytase activity and lower levels of seed PA than seeds of Williams 82 and Jack.

Fatty Acids

The fatty acid content of R8 seeds of the entries from the 2006 and 2007 Yield Component Study are presented in Table 4. Fatty acids among the entries were variable between 2006 and 2007. Palmitic (16:0) and oleic (18:1) acid levels were higher in 2007 than in 2006. Levels of linoleic (18:2) and linolenic (18:3) acids were higher in 2006 than 2007, and there was no difference in stearic acid (18:0) between 2006 and 2007. Seeds of CX1834-1-3 were found to have more 16:0 than the other entries in 2006 and 2007. Seeds of Williams 82, Jack, and CAPPA did not differ in 16:0 either year. Entry seed 18:0, 18:1, 18:2, and 18:3 levels were variable across the two years, but seeds of CAPPA and Jack did not differ in the amounts of the aforementioned seed fatty acids in 2006 or

2007, indicating that CAPPA was equal to its parent line, Jack; i.e. the transformation of CAPPA for the production of high phytase did not appear to affect the fatty acid profile of these major fatty acids of any subsequent seeds. This was expected, as the transformation of Jack to CAPPA was targeted and not foreseen to affect fatty acid content of the seeds.

Macronutrient and Micronutrient Analyses

Results of macronutrient and micronutrient analyses of R8 seeds of the four entries from the 2006 and 2007 Yield Component Study are presented in Tables 5 and 6, respectively. Mean macronutrient and micronutrient levels were significantly greater in 2006 than 2007, with the exceptions of Fe and B, which were greater in 2007 than in 2006. Seeds of Jack contained less N than seeds of CAPPA in 2006, but there was no difference in seed N between the two entries in 2007. The entries did not differ in their amount of seed P either year, but P was the only seed nutrient to respond in the manner. Seeds of CAPPA had significantly less K than the other entries in 2006. In 2007, K levels in seeds of CAPPA did not differ from levels in seeds of CX1834-1-3, but was lower than seeds of Jack. Seeds of Williams 82 contained less Ca than the other entries in 2006 and 2007. Seeds of CAPPA and Jack did not differ in their mean levels of Ca, Mg, Zn, Fe, Mn, Cu, or B in 2006 or 2007, but seeds of all four entries were variable in their macro and micronutrients composition. While Fe levels were higher in the second study year, B levels averaged across the four entries were 55.7 mg kg^{-1} in 2007 compared to 33.6 mg kg^{-1} in 2006, with an $LSD_{0.05}$ of 4.4. This difference was not expected, and further research is necessary to test physiological effects of B levels in entry seed.

The mean protein content (based on N content*6.25) of entry seed was greater in 2006 than 2007, and was variable between the two years. In 2006, Jack contained lower protein content than CAPPA and CX1834-1-3, but in 2007 CX1834-1-3 and Williams 82 were lower than Jack. CAPPA and Jack did not differ in protein in 2007.

Spear and Fehr (2007) compared CX1834, from which the mutagenized low phytate line in this study was derived, to a normal phytate soybean line (B019) and found no difference in protein content. Wilcox and Shibles (2001) tested 43 soybean lines over 3 environments, and found protein levels varied significantly among the lines and environments. Variation in macro and micro-nutrient content and protein among the four entries are also consistent with results from Raboy, *et al*. (1984), who also observed variation in seed total P, Zn, Ca, Mg, and protein among soybean lines. These data indicate CAPPA was equal to its parent line, Jack; i.e. the transformation of CAPPA for high phytase did not appear to affect nutrient levels or protein content of transformed seeds. This was expected, as the transformation of Jack to CAPPA was targeted and not foreseen to affect macro or micronutrient content of the seeds.

Yield Component Study Vigor Tests

Mean total percent standard germination of seeds of the four entries from the 2006 and 2007 Yield Component Study is presented in Table 7. Standard germination across the entries was considerably higher in 2006 (mean 94%) than 2007 (mean 65%). Seeds of the entries not utilized in 2006 germination testing were stored and planted in field studies in 2007. Field emergence of the 2007 entry seeds was considerably lower than the standard germination tests on the 2006 harvested seeds predicted. The

emergence of Williams 82 seedlings in 2007 (Table 1) was 70%; while standard germination was 99%, 20% lower than in the standard germination test (Table 7). Seedling emergence of Jack, CAPPA, and CX1834-1-3 were approximately 40% lower than their respective germination percentages in the standard germination test. The standard germination test is widely agreed upon as an unacceptable sole indicator of field emergence (TeKrony and Egli, 1977; Yaklich and Kulik, 1979; Egli and TeKrony, 1995). These data support that conclusion. Standard germination of the entries was high in 2006 (Table 7), and in the range considered commercially acceptable for high vigor seed (Egli and TeKrony, 1995), despite the fact that the parental seeds showed poor field emergence (Table 1). Field emergence of Williams 82 seedlings was 41% higher in 2007 than 2006 (Table 1), but the standard germination percentage of 2007 produced seed was 11% lower than 2006 produced seed. Field emergence of Jack seedlings was 29% higher in 2007 than 2006 (Table 1), but the standard germination percentage of seed produced in 2007 was 48% lower than seed produced in 2006 (Table 7). Field emergence of CAPPA seedlings was 23% higher in 2007 than 2006 (Table 1), but standard germination percentage of seed produced in 2007 was 59% lower than seed produced in 2006 (Table 7). Field emergence of CX1834-1-3 seedlings in 2007 was 30% higher than in 2006 (Table 1), but seeds of CX1834-1-3 produced in 2006 or 2007 were not different in their standard germination, with 98% of the seeds produced in each year germinating. CX1834-1-3 seedlings have been reported to show decreased vigor (Hulke, *et al*., 2004; Oltmans *et al*., 2005), but a decreased vigor for this entry was not observed in these experiments.

Percent germination following accelerated aging was greater in 2006 than 2007 for all entries except CX1834-1-3 (Table 7). Jack and Williams 82 did not differ in germination following accelerated aging (mean 95%) in 2006, and they exhibited higher germination than CAPPA and CX1834-1-3 (mean 29%), which did not differ. Williams 82 and Jack exhibited significantly higher germination following accelerated aging than CAPPA and CX1834-1-3 in 2006 and 2007 (Table 7). Germination following accelerated aging is reported to provide a more accurate assessment of field performance than standard germination alone (TeKrony and Egli, 1977; Kulik and Yaklich, 1982). The germination results following accelerated aging of seeds produced in 2006 did not predict 2007 field emergence (Tables 7 and 1, respectively), but results indicated that general vigor, and seeds of Williams 82, Jack, and CX1834-1-3 exhibited greater field emergence than seeds of CAPPA, although emergence was variable among the entries. The emergence difference between Jack seedlings and CAPPA seedlings in 2007 was minor, but significant. There were no differences in field emergence among all entries in 2006 (Table 1). The year to year variability of the field environment indicates further testing is needed to confirm these observations, and these emergence results may not exhibit the typical relationship observed with accelerated aging testing. More research is needed before a conclusion can be reached as to the reliability of the accelerated aging test to predict field emergence of CAPPA.

Laboratory Seed Vigor Experiments

Vigor and viability test results of the entries utilized in laboratory seed vigor experiments were compared to their field emergence in the Seedling Dry Mass study and are presented in Table 8. Seeds of Williams 82, Jack, CAPPA, and CX1834-1-3 did not

differ in mean standard germination percentages. The entries also did not differ statistically in germination following accelerated aging, although seed of Williams 82 and Jack exhibited higher germination than seeds of CAPPA and CX1834-1-3. Mean germination percentages among the entries were low and variable, resulting in a high $LSD_{0.05}$. Williams 82 seedlings exhibited greater emergence following cold testing than the other entries. CAPPA did not differ from Jack or CX1834-1-3, but CX1834-1-3 was significantly lower in germination than Jack following the cold testing. Results of tetrazolium and electrical conductivity testing were similar among all entries.

Standard germination and tetrazolium staining are not reliable vigor tests to estimate field emergence (TeKrony and Egli, 1977; Yaklich and Kulik, 1979; Egli and TeKrony, 1995). These tests estimate viability, and indicated high viability for all entries in this study, but the tests do not account for stressful environmental conditions often present in the field environment, and could overestimate the potential of seedling emergence in field plantings. The four entries demonstrated high viability following tetrazolium staining and high standard germination (Table 8), yet their field emergence was consistently lower than these two tests would have predicted (Table 8). Laboratory vigor standard germination data agree with the Yield Component Study standard germination data (Table 7) and the combined data indicated a potential emergence greater than what was exhibited in the field (Table 8). These results support the concept that two seed lots with similar standard germination under laboratory conditions, may perform differently in field environments due to seed vigor differences (as reviewed by TeKrony and Egli, 1991). These data do not support the findings of Spear and Fehr (2007) that standard germination is an effective predictor of reduced field emergence of CX1834

derived soybean lines. In these studies, CX1834-1-3 consistently exhibited high standard germination (Tables 7 and 8, respectively), but reduced field emergence, indicating seeds likely germinated but failed to emerge. Hamman *et al*. (2002) concluded that lack of germination did not account for poor soybean emergence, rather post germinative seedling growth was more important in accessing high vigor seed lots.

Seeds of the four entries exhibited no differences in electrical conductivity (Table 8). This finding may indicate electrical conductivity is not accurate in predicting field emergence, which supports research conducted by Vieira, *et al*. (1999) that under adverse field conditions during the time of germination and emergence, electrical conductivity becomes less reliable as a predictor of soybean seedling emergence.

The cold test and the accelerated aging test are reported to provide better estimates of field emergence. Both test seeds in stressful conditions before transferring the seed to non-stressful conditions for germination testing. Each test employs different stresses, i.e. the accelerated aging utilizes heat and humidity, and the cold test utilizes low temperatures and field soil. The stressful environment each provides is effective for testing beyond viability. Spear and Fehr (2007) concluded that the cold and accelerated aging tests were effective in predicting the field emergence of CX1834 derived lines. The accelerated aging and cold test data in this study provided accurate assessments of seed vigor in support of Spear and Fehr's results, but also support other research (TeKrony and Egli, 1977; Yaklich and Kulik, 1979; Egli and TeKrony, 1995) that the standard germination test was not as effective in predicting field emergence as other vigor tests.

The germination results following seed pretreatment in the cold test may be the best indication of future field performance of the entries in this study (Table 8). Johnson and Wax (1978) found the cold test to be more effective than standard germination or accelerated aging for identifying low yielding seed lots, i.e., lines demonstrating poor germination and emergence, which lead to lower stand counts. The utmost setback to use of the cold test as the primary vigor test for seeds is the lack of standardization among seed testing institutions. The cold test has proven cumbersome and impossible to standardize because soil from the field environment in which the seed lot will be planted is used to mimic the field environment as closely as possible making it impossible to replicate the test conditions across laboratories. Two vital facets of a successful vigor test is simplicity and repeatability, and neither of those is conducive to the cold test. The method utilized with the cold test experiment was previously used by Vieira, *et al*. (1992), and was selected for these experiments because of its clarity and simplicity of methods used. Field soil from the plot used in the study is also recommended. While it will provide stress to seeds to use potting soil in addition to the cold temperatures, field soil provides microorganisms and textural properties that are more realistic to future post-germination, pre-emergence stress. The closest standardization possible across laboratories will likely specify uniform laboratory conditions and use of soil directly from the plot to be used for field planting.

CHAPTER 4

SUMMARY AND CONCLUSIONS

A single standardized vigor test to preclude the use of all others has not been identified (as reviewed by TeKrony and Egli, 1991). The clarity of instructions and minimal physical work involved in successful, repeatable testing is vital. Accelerated aging effectively predicted field emergence with the seed lots used in these experiments (Tables 7 and 8, respectively), and is a standardized vigor test (AOSA, 2002). The laboratory germination of the entries was consistently lower than their emergence observed in the field, but the accelerated aging test was effective in showing how seeds may perform under stressful conditions and it identified low seed vigor in CAPPA and CX1834-1-3.

Egli and TeKrony (1995) provided a concise summary of laboratory vigor tests and their use to predict future field performance of soybean seed lots. They determined the association between a laboratory vigor test to field emergence depends greatly on seedbed conditions. While the standard germination test and tetrazolium staining provide insight on seed viability, and the accelerated aging and cold tests indicate seed vigor, there is no adequate substitute for field performance. Laboratory vigor tests are tools to assist the researcher, but germination and emergence in soil is essential to an accurate assessment of a seed lot's vigor. Regulations restrict field performance testing of transgenic crops and may slow advancement of potentially competitive experimental

lines, but advancing transgenic seeds as viable alternatives to chemically mutagenized seeds must include field testing.

Field emergence, yield, and vigor test data were analyzed for Williams 82, Jack, CAPPA, and CX1834-1-3. Williams 82 consistently exhibited higher field emergence and vigor than the other entries. CX1834-1-3, the mutagenized low-phytate entry, did not perform as expected and exhibited higher field emergence and germination than anticipated, although vigor demonstrated during the vigor experiments was low as expected. Jack and Williams 82 are both commercial soybean varieties, but Jack did not perform as well as Williams 82. Results were not conclusive in the comparison between CAPPA and Jack. While field emergence was variable, CAPPA field emergence was lower than Jack. The vegetative components of Jack weighed more than CAPPA, which was unexpected. Yield, which was calculated based on an equal number of plants from each entry, showed no difference in Jack and CAPPA, as expected. It is important to note this difference. Jack grew to be bigger plants, but did not out yield CAPPA. CAPPA and Jack exhibited no difference in viability, but CAPPA may have decreased vigor. Stress during pod fill may have affected the emergence of seeds developed during stress, and CAPPA deterioration may have begun earlier than Jack. These questions require further research. CAPPA seed is consistently high in phytase activity with undetectable levels of PA, as expected, and use in livestock feed may prove economical. Because Jack did not perform as expected, this brings to question whether CAPPA exhibited lower vigor because of biological complications caused by the transformation of Jack, or because of its parental lineage. It is suggested that the cytoplasmic appA gene be inserted into another released cultivar to test the vigor in a second variety.

Table 1: Total percent emergence of soybean seedlings of four entries in Seedling Dry Mass Study in 2006 and 2007 at the Bradford Research and Extension Center, Columbia, MO.

Entry	2006	2007
	%	
Williams 82	29	70
Jack	28	57
CAPPA	20	43
CX1834-1-3	27	57
$LSD_{0.05}$	13	13

Table 2: Mean seed weight, number of seeds per plant, and yield at R8 of four soybean entries in the Yield Component Study. Seeds were produced in 2006 and 2007 at the Bradford Research and Extension Center, Columbia, MO.

	2006			2007		
Entry	Seed Weight	Seeds/ Plant	Yield	Seed Weight	Seeds/ Plant	Yield
	mg $seed^{-1}$		g seed $plant^{-1}$	mg $seed^{-1}$		g seed $plant^{-1}$
Williams 82	130.4	454	59.08	133.4	609	81.00
Jack	120.2	400	47.90	105.1	536	56.63
CAPPA	128.2	495	63.38	112.2	573	64.07
CX1834-1-3	147.8	285	41.74	139.2	540	75.66
$LSD_{0.05}$	15.1	90	14.50	13.1	199	23.29

Table 3: Total available mean P, phytic acid (PA), and phytase activity of R8 seeds of four soybean entries in the Yield Component Study. Seeds were produced in 2006 and 2007 at the Bradford Research and Extension Center, Columbia, MO.

	2006			2007		
Entry	Available P	PA	Phytase Activity	Available P	PA	Phytase Activity
	µg P mg^{-1} seed	µg PA mg^{-1} seed	µmol P min^{-1} mg^{-1} seed	µ P mg^{-1} seed	µg PA mg^{-1} seed	µmol P min^{-1} mg^{-1} seed
Williams 82	0.45	17.49	0.02	0.28	18.69	0.01
Jack	0.34	18.75	0.08	0.18	27.54	0.02
CAPPA	4.75	None detected	39.57	4.94	None detected	33.28
CX1834-1-3	4.43	4.81	0.09	3.84	4.90	0.01
$LSD_{0.05}$	0.38	1.60	4.11	0.35	9.02	5.03

Table 4: Fatty acid content of R8 seeds of four soybean entries in the Yield Component Study. Seeds were produced in 2006 and 2007 at the Bradford Research and Extension Center, Columbia, MO.

	Fatty Acids									
	2006					2007				
Entry	16:0	18:0	18:1	18:2	18:3	16:0	18:0	18:1	18:2	18:3
	$g\ kg^{-1}$									
Williams 82	102	40	202	564	92	117	43	251	523	66
Jack	105	46	205	560	85	116	47	250	522	65
CAPPA	106	44	205	563	83	119	44	231	543	64
CX1834-1-3	116	51	199	547	88	136	48	224	524	68
$LSD_{0.05}$	3	2	12	9	8	7	5	19	22	4

Table 5: Macronutrient and micronutrient analysis of R8 seeds of four soybean entries in the Yield Component Study. Seeds were produced in 2006 at the Bradford Research and Extension Center, Columbia, MO.

	Macronutrients					Micronutrients				
Entry	N	P	K	Ca	Mg	Zn	Fe	Mn	Cu	B
	$g\ kg^{-1}$					$mg\ kg^{-1}$				
Williams 82	59.6	6.2	20.5	2.9	2.3	47.18	73.25	30.05	14.73	32.39
Jack	58.0	6.3	19.7	3.7	2.6	51.10	80.53	30.35	17.95	35.45
CAPPA	61.3	6.2	18.4	3.6	2.6	52.35	77.88	30.33	15.88	36.06
CX1834-1-3	59.8	6.2	20.1	3.4	2.2	54.65	81.93	24.53	16.63	30.68
$LSD_{0.05}$	2.9	NS	0.8	0.4	0.2	4.14	6.07	3.53	3.15	4.49

Table 6: Macronutrient and micronutrient analysis of R8 seeds of four soybean entries in the Yield Component Study. Seeds were produced in 2007 at the Bradford Research and Extension Center, Columbia, MO.

	Macronutrients					Micronutrients				
Entry	N	P	K	Ca	Mg	Zn	Fe	Mn	Cu	B
	$g\ kg^{-1}$					$mg\ kg^{-1}$				
Williams 82	45.2	5.8	18.2	2.4	2.1	46.65	77.90	24.28	13.65	54.58
Jack	46.0	5.9	18.8	2.9	2.2	49.78	80.98	26.88	14.35	56.31
CAPPA	45.6	5.7	17.6	3.2	2.3	49.55	80.20	28.83	14.88	57.17
CX1834-1-3	45.3	5.8	17.8	3.4	2.3	50.13	93.03	25.10	16.80	54.65
$LSD_{0.05}$	0.8	NS	0.7	0.4	0.2	NS	8.75	2.23	1.83	NS

Table 7: Total percent standard germination and germination following accelerated aging of four entries of soybean seed at the R8 stage. Seeds were produced at the Bradford Research and Extension Center, Columbia, MO in 2006 and 2007.

	2006		2007	
Entry	Standard Germination	Accelerated Aging	Standard Germination	Accelerated Aging
	%			
Williams 82	99	93	88	85
Jack	97	97	49	60
CAPPA	84	35	25	21
CX1834-1-3	98	23	98	29
$LSD_{0.05}$	6	21	14	12

Table 8: Vigor tests, viability tests, and field emergence tests were utilized to evaluate four soybean entries. Vigor of seed utilized for laboratory vigor testing was compared to field emergence of Seedling Dry Mass Study seed.

		Entry				
Test Name	Test Type	Williams 82	Jack	CAPPA	CX1834-1-3	$LSD_{0.05}$
		%				
Standard Germination†	Viability	93	88	87	92	NS
Accelerated Aging†	Vigor	13	14	5	6	NS
Cold Test†	Vigor	28	21	15	11	6
Tetrazolium Test†	Viability	83	85	81	85	NS
Field Emergence 2006‡		29	28	20	27	NS
Field Emergence 2007†		70	57	43	57	13
		$\mu S cm^{-1} g^{-1}$				
Electrical Conductivity†	Vigor	1016	1079	1039	1096	NS

† Seeds produced at the Bradford Research and Extension Center, Columbia, MO, in 2006.

‡ Seeds produced at the Sears Greenhouse, University of Missouri, Columbia, MO, in 2006.

Table 9: Analysis of soil samples from field plots in 2006 and 2007 at Bradford Research and Extension Center, Columbia, MO.

Year	pH	N.A.†	O.M.	C.E.C.	E.C.	Nutrients										
						P	Ca	Mg	K	Na	SO_4-S	Zn	Fe	Mn	Cu	B
		meq/100g	%	meq/100g	mmhos/cm	kg ha^{-1}					ppm					
2006	6.1	1.0	2.2	14.6	0.2	41.4	4786.9	701.1	262.1	51.5	9.1	0.9	40.2	20.5	1.05	0.23
2007	6.3	1.0	1.5	10.4	0.2	35.8	3514.6	358.4	221.8	29.1	4.4	0.4	25.2	10.7	1.05	0.23

†N.A., Neutralizable Acidity; O.M., Organic Matter; C.E.C., Cation Exchange Capacity; E.C., Electrical Conductivity

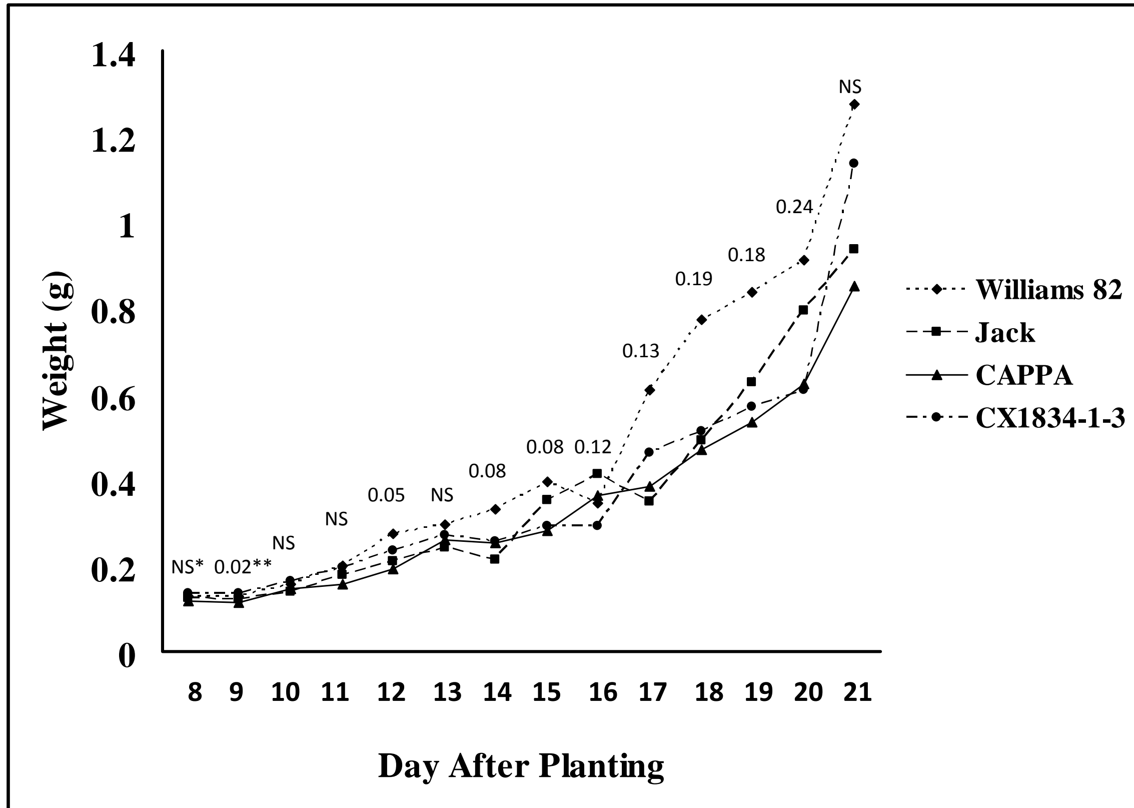
Table 10: Managements applied to two field studies at the Bradford Research and Extension Center, Columbia, MO, in 2006 and 2007.

Year	Study†	Pre-emergent Herbicides	Planted	Sample Timeline	Irrigation
2006	SDMS	2.3 L/hectare Dual II Magnum (82.4% *S*-Metolachlor)	8 June	NA‡	As needed
	YCS	42 g/hectare FirstRate (84% Chloransulam-methyl)	9 June	R5-R8 (21 Aug.-9 Oct.)	As needed
2007	SDMS	2.3 L/hectare Dual II Magnum (82.4% *S*-Metolachlor)	4 June	8 DAP-21 DAP (12 June-25 June)	As needed
	YCS	196 g/hectare Scepter 70 DG (70% Imazaquin)	5 June	R5-R8 (15 Aug.-27 Sept.)	As needed

† SDMS, Seedling Dry Mass Study; YCS, Yield Component Study

‡ Poor emergence due to severe crusting of soil.

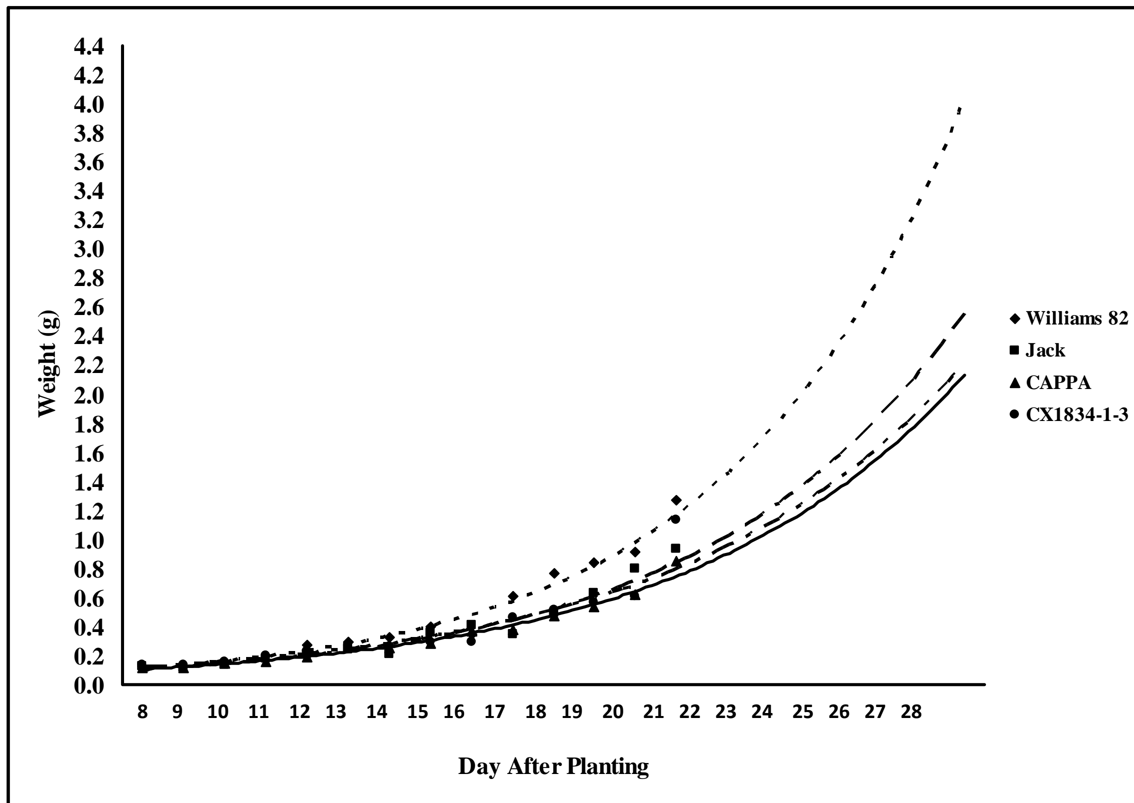
Figure 1: Cumulative above ground seedling dry mass of four soybean entries grown at the Bradford Research and Extension Center, Columbia, MO in 2007. Seedlings were harvested daily for 14 days consecutively starting 8 days after planting (DAP).



* No significant difference in seedling dry mass among the four entries

** $LSD_{0.05}$

Figure 2: Cumulative above ground seedling dry mass of four soybean entries grown at the Bradford Research and Extension Center, Columbia, MO in 2007. Seedlings were harvested daily for 14 days consecutively starting 8 days after planting (DAP). The predictive growth for the 7 days following sampling (22-28 DAP) are given to illustrate growth potential of the entries. Predictive growth equations for entries listed below the figure.



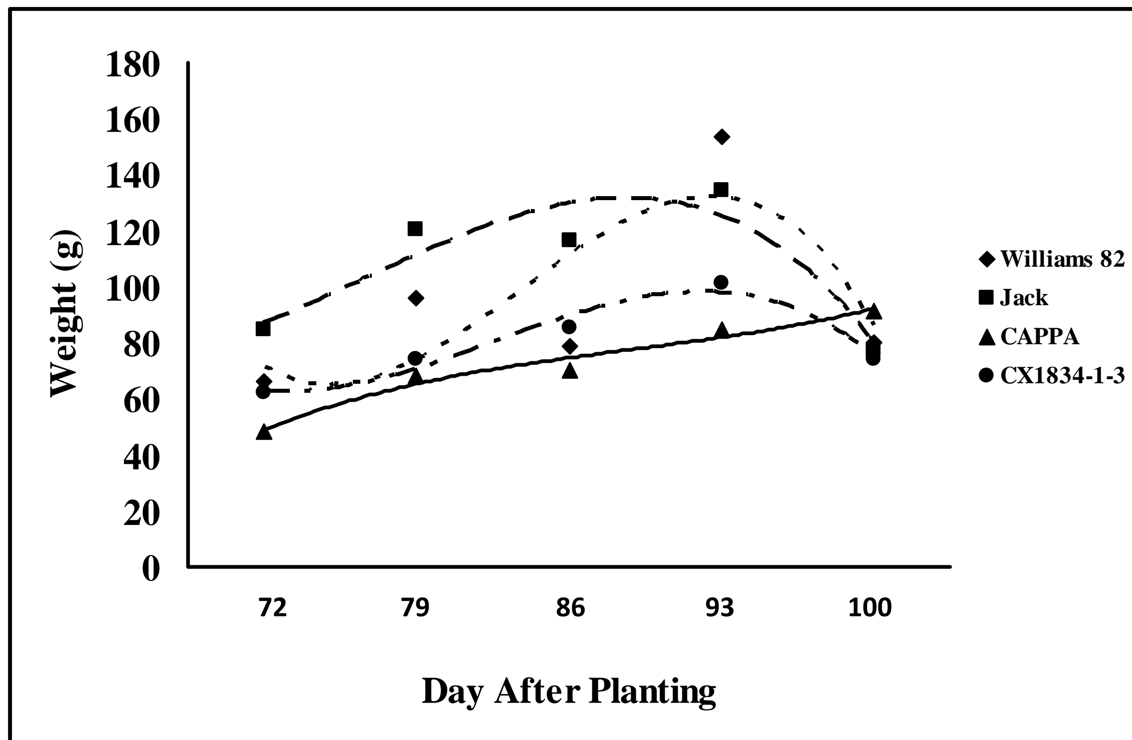
Williams 82: $y = 0.0977e^{0.1771x}$, $R^2 = 0.9748$

Jack: $y = 0.0915e^{0.1585x}$, $R^2 = 0.9657$

CAPPA: $y = 0.092e^{0.1496x}$, $R^2 = 0.9861$

CX1834-1-3: $y = 0.1045e^{0.1453x}$, $R^2 = 0.9448$

Figure 3: Mean total plant weight, excluding roots, of four soybean entries on five weekly sampling dates commencing at R5 (72 DAP) and concluding at R7 (107 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2006. Regression equations for entries are listed below the figure.



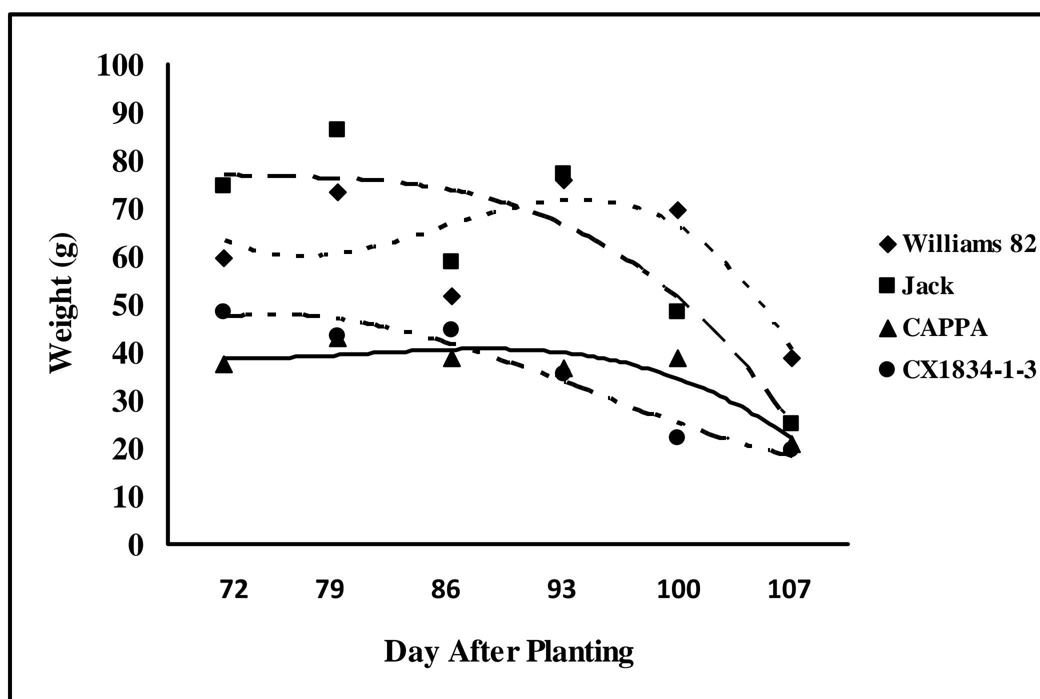
Williams 82: $y = -8.4739x^3 + 68.147x^2 - 142.65x + 154.58$, $R^2 = 0.5681$

Jack: $y = -2.9386x^3 + 14.857x^2 + 0.1258x + 75.226$, $R^2 = 0.8472$

CAPPA: $y = 0.8412x^3 - 8.5817x^2 + 36.246x + 20.437$, $R^2 = 0.9687$

CX1834-1-3: $y = -3.574x^3 + 27.03x^2 - 48.463x + 88.284$, $R^2 = 0.9523$

Figure 4: Total above ground vegetative weight of four soybean entries on six sampling dates commencing at R5 (72 DAP) and concluding at R7 (107 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2006. Regression equations for entries are listed below the figure.



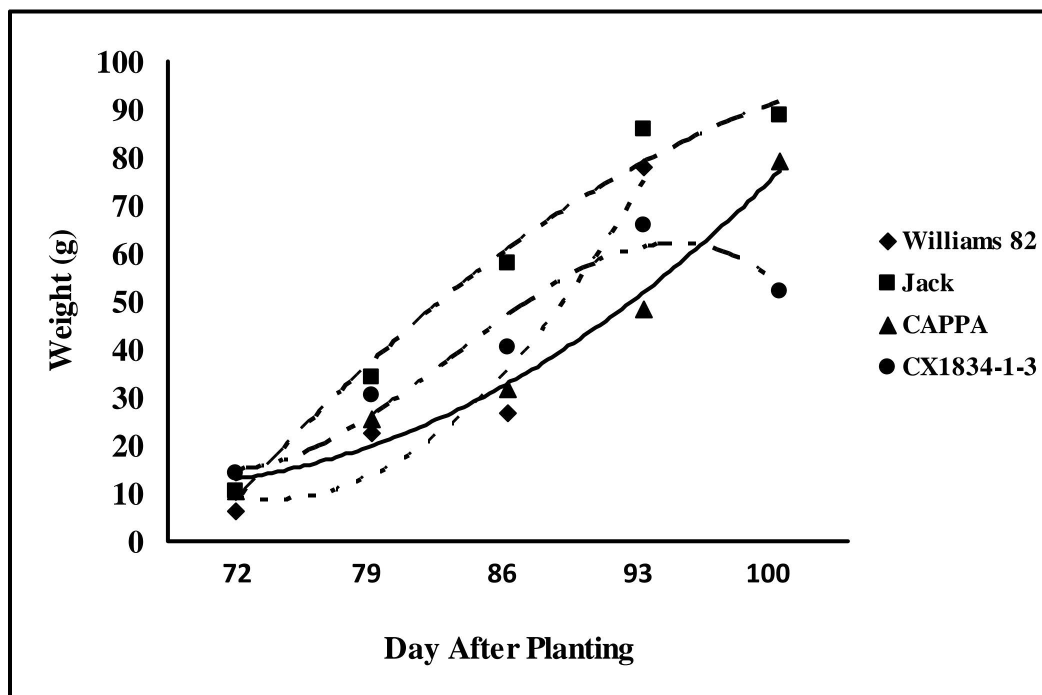
Williams 82: $y = -1.6127x^3 + 14.059x^2 - 33.677x + 84.613$, $R^2 = 0.5881$

Jack: $y = -0.5113x^3 + 2.1711x^2 - 3.6166x + 78.911$, $R^2 = 0.8188$

CAPPA: $y = -0.441x^3 + 3.0037x^2 - 5.4312x + 41.751$, $R^2 = 0.8435$

CX1834-1-3: $y = 0.3765x^3 - 4.7697x^2 + 11.406x + 40.28$, $R^2 = 0.951$

Figure 5: Total reproductive weight of four soybean entries on five sampling dates commencing at R5 (72 DAP) and concluding at R7 (100 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2006. Regression equations for entries are listed below the figure.



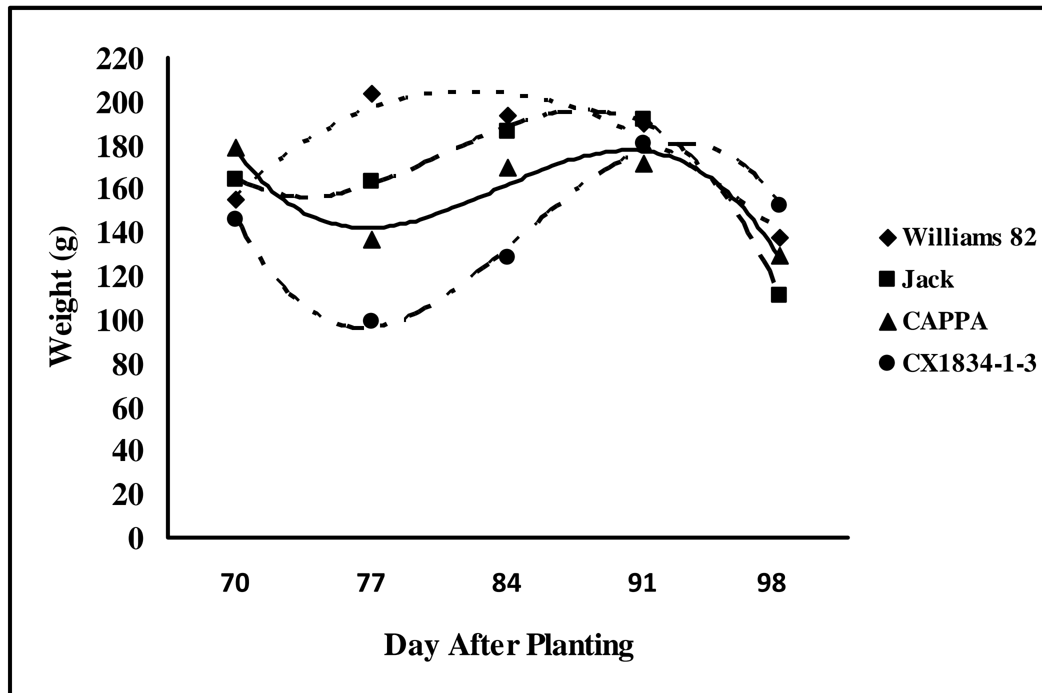
Williams 82: $y = 8.785x^2 - 22.015x + 22.566$, $R^2 = 0.9407$

Jack: $y = -2.6876x^2 + 36.927x - 25.773$, $R^2 = 0.9819$

CAPPA: $y = 3.0553x^2 - 2.308x + 12.309$, $R^2 = 0.9798$

CX1834-1-3: $y = -2.6993x^3 + 21.076x^2 - 33.237x + 29.987$, $R^2 = 0.9465$

Figure 6: Total above ground weight of four soybean entries on five sampling dates commencing at R5 (70 DAP) and concluding at R7 (98 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2007. Regression equations for entries are listed below the figure.



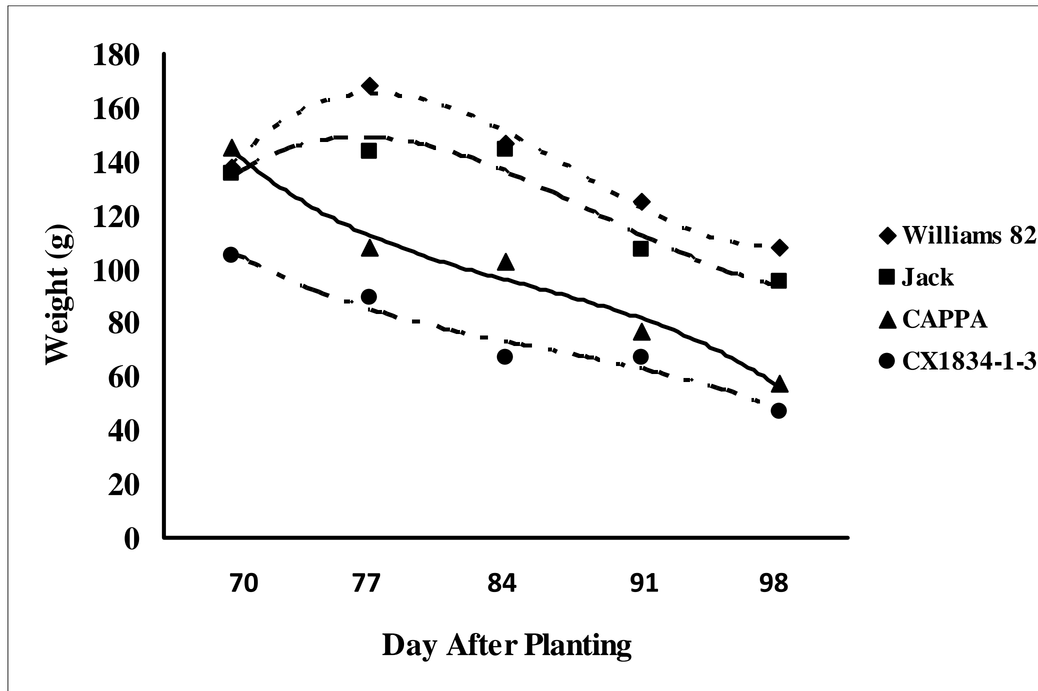
Williams 82: $y = 0.8809x^3 - 21.924x^2 + 100.03x + 77.256$, $R^2 = 0.9327$

Jack: $y = -9.1333x^3 + 69.604x^2 - 147.88x + 252.27$, $R^2 = 0.9981$

CAPPA: $y = -9.9897x^3 + 87.639x^2 - 228.53x + 328.4$, $R^2 = 0.9375$

CX1834-1-3: $y = -12.985x^3 + 121.07x^2 - 322.39x + 360.9$, $R^2 = 0.9902$

Figure 7: Total above ground vegetative weight of four soybean entries on five sampling dates commencing at R5 (70 DAP) and concluding at R7 (98 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2007. Regression equations for entries are listed below the figure.



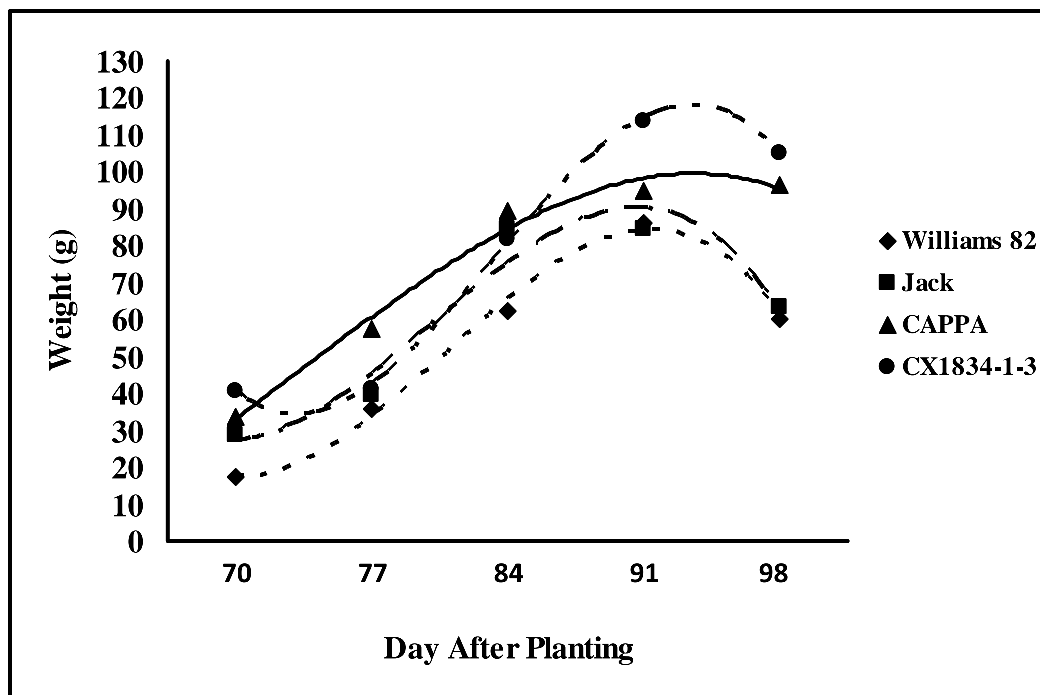
Williams 82: $y = 4.6594x^3 - 48.812x^2 + 140.99x + 41.205$, $R^2 = 0.9831$

Jack: $y = 2.7328x^3 - 30.123x^2 + 85.879x + 75.782$, $R^2 = 0.936$

CAPPA: $y = -2.1479x^3 + 20.329x^2 - 77.376x + 203.09$, $R^2 = 0.9798$

CX1834-1-3: $y = -1.1028x^3 + 10.955x^2 - 46.021x + 142.14$, $R^2 = 0.9667$

Figure 8: Total reproductive weight of four soybean entries on five sampling dates commencing at R5 (70 DAP) and concluding at R7 (98 DAP). Plants were grown at the Bradford Research and Extension Center, Columbia, MO in 2007. Regression equations for entries are listed below the figure.



Williams 82: $y = -4.8699x^3 + 37.235x^2 - 61.684x + 46.987$, $R^2 = 0.9936$

Jack: $y = -4.6063x^3 + 33.762x^2 - 51.122x + 49.385$, $R^2 = 0.9424$

CAPPA: $y = -1.0602x^3 + 4.4711x^2 + 21.671x + 7.9165$, $R^2 = 0.9838$

CX1834-1-3: $y = -6.7054x^3 + 58.377x^2 - 126.41x + 115.39$, $R^2 = 0.9991$

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