Soybean [Glycine max (L.) Merr.] agronomic performance, seed characteristics, and aconitase isozymes variability in response to environmental stimuli

Grace Kaudzu
Iowa State University

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Soybean [Glycine max (L.) Merr.] agronomic performance, seed characteristics, and aconitase isozymes variability in response to environmental stimuli

by

Grace Kaudzu

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Crop Production and Physiology (Seed Science)

Program of Study Committee:
A. Susana Goggi, Co-Major Professor
Russell E. Mullen, Co-Major Professor
Silvia R. Cianzio
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Kenneth J. Moore

The student author and the program of study committee are solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2017

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DEDICATION

I dedicate this dissertation to my loving husband Richard Joseph Kaudzu, and our two lovely daughters, Nthanda (Morning Star) and Chimango (Unity), for being my greatest intercessors and supporters. May the good Lord bless you beyond measure. Also, I dedicate this work to Dr. Reid G. Palmer (late), who had great plans for this project but never saw its progress. I thank God for the two years we interacted.

“Do not be anxious about anything, but in every situation, by prayer and petition, with thanksgiving, present your requests to God. And the peace of God, which transcends all understanding, will guard your hearts and your minds in Christ Jesus” Philippians 4:6-7.
# TABLE OF CONTENTS

**LIST OF FIGURES** ........................................................................................................ vi
**LIST OF TABLES** ........................................................................................................ vii
**ACKNOWLEDGMENTS** ............................................................................................... ix
**ABSTRACT** ................................................................................................................... xii
**CHAPTER 1. GENERAL INTRODUCTION** ................................................................. 1

- Epigenetic, genetic, and morphological responses to environmental stimuli ..... 2
- Heat stress on agronomic performance and seed characteristics ......................... 8
- Defoliation stress on agronomic performance and seed characteristics ............. 9
- Dissertation organization ....................................................................................... 11
- References ............................................................................................................... 11

**CHAPTER 2. SOYABEAN AGRONOMIC PERFORMANCE AND SEED CHARACTERISTICS IN RESPONSE TO PLANT POPULATION AND SIMULATED HAIL** ............................................. 17

- Abstract .................................................................................................................. 17
- Introduction ........................................................................................................... 18
- Materials and methods ......................................................................................... 21
  - Plant material and seed source ....................................................................... 21
  - Wide-spaced HC and CR planting designs ..................................................... 22
  - Simulated hail treatment .............................................................................. 22
- Location ................................................................................................................ 23
- Data collection ...................................................................................................... 23
- Data analysis ........................................................................................................ 25
- Results ................................................................................................................... 26
- Discussion ............................................................................................................ 28
- Conclusion ........................................................................................................... 32
- Acknowledgements ............................................................................................. 33
- References ............................................................................................................ 33
## CHAPTER 3. AGRONOMIC PERFORMANCE AND SEED CHARACTERISTICS IN ‘JACK’ VARIANTS EXPOSED TO ENVIRONMENTAL STIMULI

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>45</td>
</tr>
<tr>
<td>Introduction</td>
<td>46</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>48</td>
</tr>
<tr>
<td>Plant material and seed source</td>
<td>48</td>
</tr>
<tr>
<td>Wide-spaced HC planting design</td>
<td>50</td>
</tr>
<tr>
<td>Modified AA</td>
<td>51</td>
</tr>
<tr>
<td>Seed increase</td>
<td>51</td>
</tr>
<tr>
<td>Location</td>
<td>51</td>
</tr>
<tr>
<td>Data Collection</td>
<td>52</td>
</tr>
<tr>
<td>Data analysis</td>
<td>53</td>
</tr>
<tr>
<td>Results</td>
<td>54</td>
</tr>
<tr>
<td>Wide-spaced HC planting design</td>
<td>54</td>
</tr>
<tr>
<td>Modified AA</td>
<td>56</td>
</tr>
<tr>
<td>Discussion</td>
<td>60</td>
</tr>
<tr>
<td>Conclusion</td>
<td>66</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>66</td>
</tr>
<tr>
<td>References</td>
<td>66</td>
</tr>
</tbody>
</table>

## CHAPTER 4. ACONITASE-4 ALLELE STABILITY IN SOYBEAN ‘JACK’ VARIANTS EXPOSED TO ADDITIONAL ENVIRONMENTAL STIMULI

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>79</td>
</tr>
<tr>
<td>Introduction</td>
<td>80</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>83</td>
</tr>
<tr>
<td>Plant material and seed source</td>
<td>83</td>
</tr>
<tr>
<td>Wide-spaced HC planting design</td>
<td>85</td>
</tr>
<tr>
<td>Modified AA</td>
<td>86</td>
</tr>
<tr>
<td>Seed sample collection from wide-spaced HC planting design</td>
<td>86</td>
</tr>
<tr>
<td>Seed sample collection from modified AA</td>
<td>87</td>
</tr>
<tr>
<td>Aconitase isozyme test</td>
<td>87</td>
</tr>
<tr>
<td>Seed germination and cotyledon sampling</td>
<td>87</td>
</tr>
<tr>
<td>Sample homogenization and electrophoresis</td>
<td>88</td>
</tr>
<tr>
<td>DNA analysis</td>
<td>89</td>
</tr>
<tr>
<td>Leaf sample collection and DNA extraction</td>
<td>89</td>
</tr>
<tr>
<td>PCR</td>
<td>89</td>
</tr>
<tr>
<td>DNA sequencing, alignment and data analysis</td>
<td>90</td>
</tr>
<tr>
<td>Results</td>
<td>91</td>
</tr>
<tr>
<td>Aconitase isozyme pattern of seeds from wide-spaced HC planting design</td>
<td>91</td>
</tr>
<tr>
<td>Aconitase isozyme pattern of seeds from the modified AA treatment</td>
<td>92</td>
</tr>
<tr>
<td>The Aco4 gene (Glyma.11g080600) sequencing</td>
<td>92</td>
</tr>
<tr>
<td>Discussion</td>
<td>93</td>
</tr>
<tr>
<td>Conclusion</td>
<td>98</td>
</tr>
</tbody>
</table>
Acknowledgements............................................................................................................. 99
References .......................................................................................................................... 100

CHAPTER 5. GENERAL CONCLUSIONS............................................................................. 111
References .......................................................................................................................... 114
LIST OF FIGURES

CHAPTER 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Relationship between; (A) plant height and 100-seed weight, (B) plant height and yield in progenies of ‘BSR 101’ from plants in wide-spaced HC design across simulated hail treatment</td>
<td>42</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Simulated hail treatment imposed on ‘BSR 101’ and ‘Jack’ cultivars at V4-V5 stage in 2011 planted in a wide-spaced HC planting design and conventional rows (CR)</td>
<td>43</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Seed Source for 2013 and 2014 agronomic performance and seed characteristics study</td>
<td>44</td>
</tr>
</tbody>
</table>

CHAPTER 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Seed source for cultivar ‘Jack’ variants for agronomic performance and seed characteristics experiment conducted from 2013 to 2015</td>
<td>77</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Relationship between mean 100-seed weight and seed yield in progenies of ‘Jack’ variants from; (A) wide-spaced HC design, and (B) modified AA before planting in CR in 2015</td>
<td>78</td>
</tr>
</tbody>
</table>

CHAPTER 4

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Alignment of protein sequences from Aco4-&lt;b&gt;bb&lt;/b&gt; and Aco4-&lt;i&gt;cc&lt;/i&gt; samples</td>
<td>108</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Seed source for the stability of aconitase isozyme in ‘Jack’ variants experiment from 2013 to 2016</td>
<td>110</td>
</tr>
</tbody>
</table>
LIST OF TABLES

CHAPTER 2

Table 1  Means for planting pattern (wide-spaced HC planting design
and CR planting) for days to maturity, plant height,
100-seed weight, standard germination, seed vigor, and oil content
in ‘BSR 101’ across simulated hail treatment ........................................ 39

Table 2  Means for the interaction effect between planting pattern and simulated
hail treatment for seed yield and protein content in progenies of
‘BSR 101’ ........................................................................................................ 39

Table 3  Means for plant stand, maturity, plant height, 100-seed weight, standard
germination, seed vigor, seed oil and seed protein content in ‘BSR 101’
progenies from non-simulated and simulated hail across planting
patterns ........................................................................................................ 40

Table 4  Means for plant stand, maturity, plant height, 100-seed weight, standard
ergamation, seed vigor, seed oil and protein content in progenies of
‘Jack’ cultivar from HC planting design and CR planting across
simulated hail treatment ............................................................................ 40

Table 5  Means for plant stand, maturity, plant height, seed yield, 100-seed
weight, standard germination, seed vigor, seed oil and protein
content in progenies of ‘Jack’ cultivar from non-simulated hail
and simulated hail across planting patterns ............................................. 41

CHAPTER 3

Table 1  Means for plant stand, plant height, maturity, yield, seed weight,
standard germination, seed vigor, oil, and protein content of progenies
of three soybean genotypes from wide-spaced HC planting design ........ 72

Table 2  Means for plant stand, plant height, maturity, yield, seed weight,
standard germination, seed vigor, oil, and protein content of progenies
of entries of the three soybean genotypes from wide-spaced HC
planting design ................................................................................................ 73

Table 3  Means for plant stand, plant height, maturity, yield, seed weight,
standard germination, seed vigor, oil, and protein content of progenies
of three soybean genotypes from modified AA ....................................... 74
Table 4  Means for plant stand, plant height, maturity, yield, seed weight, standard germination, seed vigor, oil, and protein content of progenies of entries of three soybean genotypes from modified AA.......................... 75

Table 5  Means for plant stand, plant height, maturity, yield, 100-seed weight, standard germination, seed vigor, oil, and protein content of AA or no AA treatment across genotypes ................................................................. 76

CHAPTER 4

Table 1  Seed source for cultivar ‘Jack’ Aconitase-4 (Aco4) isozyme experiment from 2008 to 2015........................................................................................................................................................................... 105

Table 2  Forward and reverse primer, PCR product size, start and end position of the primer pairs used for PCR and sequencing Glyma.11g080600 of the new cultivar ‘Jack’ Aco4 variants and original ‘Jack’ cultivar generated from wide-spaced HC planting design.............................................. 106

Table 3  Single nucleotide polymorphisms (SNPs) identified among six soybean entries/variants and their location on Glyma.11g080600 gene sequenced in 2016........................................................................................................... 107
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ABSTRACT

Environmental stimuli can generate intra-cultivar genetic variation in nearly homozygous soybean cultivars. This genetic variation may be useful for enhancing genetic diversity and developing new cultivars. Soybean plants exposed to multiple environmental stimuli produce seed variants with different phenotypic and genotypic characteristics. When these variants are selfed, the variation is heritable and stable. Seed produced in plants subjected to wide-spaced honeycomb (HC) planting design, natural hail and to a modified accelerated aging (AA) treatment at planting has exhibited intra-cultivar genetic variation. However, the effect of a single environmental stimulus on agronomic performance, seed characteristics and stability of variants from a soybean plant and its progeny had not been evaluated. The main objective of the present study was to evaluate agronomic performance, seed characteristics and aconitase-4 (Aco4) isozymes allele variability in progenies of soybean cultivars and variants exposed to environmental stimuli. The stimuli included the wide-spaced HC planting design, simulated hail and modified AA. Progenies from soybean plants grown in wide spaced HC planting design had greater variation in agronomic performance and seed characteristics than those from conventional-row planting and modified AA. Simulated hail treatment did not have a significant effect on agronomic performance. Progenies from variants had greater variability and performed better for most agronomic performance and seed characteristics than those from soybean original cultivar. New double and single Aco4 isozyme allele switching were identified in one seed within a three-seeded pod of the variants grown in a wide space HC planting design. DNA
sequence data of the *Aco4* gene (*Glyma.11g080600*) of these new variants indicated single nucleotide polymorphisms (SNPs) substitutions in the exons, which resulted in amino acid substitution and production of slightly different proteins. These studies confirmed that the wide-spaced HC planting design is a valuable tool for generating intra-cultivar genetic and phenotypic variation in soybean cultivars. Plant breeders could use wide-spaced HC planting design to induce novel genetic and phenotypic variation for new cultivar development.
CHAPTER 1. GENERAL INTRODUCTION

According to the United Nation World Population Prospects report (2015), world population is predicted to increase by one billion people within the next 15 years, reaching 9.7 billion by 2050. This prediction has led to a call for an increase in crop productivity to feed the growing population (Tester and Langridge, 2010). Coupled with this population growth, crop production has faced serious challenges due to climate change that has increased fluctuating temperatures and rainfall, reduced sustainability for annual cropping systems, and fostered the emergence of new abiotic and biotic stresses (Tester and Langridge, 2010; Prasanna et al., 2013). Therefore, increasing crop production requires advanced and faster development of technologies, such as crop varieties, that are resilient to changing climate. Development of resilient cultivars requires adequate and appropriate genetic diversity, which has been reported to be dwindling due to fewer germplasm introductions, domestication, and inbreeding obstacles especially in self-pollinated crops (Rasmusson and Phillips, 1997; Buckler and Thornsberry, 2002; Hyten et al., 2006; Tester and Langridge, 2010).

It is estimated that 45,000 soybean landraces have been collected and are being kept in genebanks worldwide (Carter et al., 2004). However, only 80 landraces account for 99% of the parentage in North American cultivars, which produces over 47% of the world’s soybean production (FAO statistics, 2014). Of the 80 landraces, 26 contribute 90% of the parentage with the rest contributing only 1% each (Gizlice et al., 1994; Carter et al., 2004). According to Carter et al. (2004), two of the five factors that have contributed to this limited use of genetic diversity in soybean are: agronomic inferiority of the
germplasm accessions compared to commercial cultivars, and inadequate knowledge of
the scope and nature of the alleles and their relationship with those already introgressed
into commercial cultivars (Carter et al., 2004). Therefore, identifying new sources of
genetic diversity within elite cultivars and exploitation of desired traits in landraces and
wild relatives are critical in addressing the upcoming challenges in meeting world food
needs (Tester and Langridge, 2010).

There is increasing evidence of new intrinsic genetic variation that can be
generated in nearly homozygous cultivars that has significant economic value (Rawlings
et al., 1958; Fasoula and Boerma, 2005, 2007; Yates et al., 2012; Hopkins et al., 2013;
Espinosa et al., 2015). This new intrinsic variation has been attributed to the instability of
the genome when exposed to environmental stimuli or stress (McClintock, 1984; Roth et
al., 1989; Walbot and Cullis, 1985; Ceccarelli et al., 1997; Casacuberta and
Puigdomènech, 2000). The stimuli include but are not limited to plant density, high/low
temperatures, UV-radiation, salt, drought, flooding, hail, nutrients and biotic stresses,
especially pathogen infection (Fasoula, 1995; Pencika et al., 2009; Krasensky and Jonak,
2012; Kravets et al., 2012). Due to their sessile nature, plants develop mechanisms to
adapt or respond to environmental changes, and these mechanisms could be genetic,
epigenetic, morphological, as well as physiological in nature (Walbot and Cullis, 1985;
Molinier, 2006; Boyko and Kovalchuk, 2011; Migicovsky et al., 2014).

**Epigenetic, genetic, and morphological responses to environmental stimuli**

Environmentally induced genetic variation was observed in plants arising from
tissue culture that could not be traced to original parent in flax (Durrant, 1962; Evans et
al., 1966; Cullis et al., 1999; Cullis, 2005). The variant plants, called ‘genotrophs’, had
different plant weight at maturity when grown under normal conditions (Durrant, 1962). Analysis of DNA content showed that large genotrophs had 16% more DNA than the small genotrophs, but the amount of DNA kept reducing in subsequent generations although the phenotype was stable (Evans et al., 1966). Further DNA analysis of the genotrophs indicated random amplified polymorphic DNA (RAPD) polymorphisms across the genome, which was suggested to be due to DNA amplification or deletions (Cullis, et al., 1999).

Brettell et al. (1986) identified one stable variant of alcohol dehydrogenase (Adhl), from 645 maize tissue cultures that had a different electrophoretic mobility than the original parent. Sequencing of the Adhl gene indicated a single base change in the exon that resulted in amino acid substitution of valine for glutamic acid, and thus a different protein with a different mobility. In the same crop, Brettell and Dennis (1991) found that tissue culture activated transposable element Activator (Ac) induced DNA methylation changes that were stable and heritable through meiosis.

Within cultivar variation for seed oil, protein, seed weight and other traits was observed in single plants selected from a wide-spaced planting design, termed honeycomb (HC), in Benning, Cook and Haskell soybean cultivars (Fasoula and Boerma, 2005, 2007). DNA analysis with simple sequence repeat (SSR) markers showed that the majority of the variation was due to residual heterozygosity that could be traced back to original parents and foundation seed source, but 2, 6 and 2 variant bands in Benning, Cook and Haskell cultivars, respectively, were attributed to de novo genetic variation (Yates et al., 2012). Evaluation of these single plants resulted in registration of a total of
19 lines with stable oil and protein contents that could be useful in plant breeding programs (Fasoula et al., 2007a, 2007b, 2007c).

Using the same HC planting design, within cultivar single plant selection was successful in other crops, such as tomato (Christakis and Fasoulas, 2002), maize inbred lines (Tokatlidis, 2000), cotton (Tokatlidis, et al., 2008, 2011), and wheat (Fasoula, 1990; Tokatlidis et al., 2004, 2006). The HC planting design allows single plants to grow in hills at ultra-wide spacing to reduce interplant competition for growth resources (Fasoula and Fasoula, 1995; Fasoula and Tokatlidis, 2012). This reduced competition allows plants to exploit soil heterogeneity, genotype by environmental interaction, and to express phenotypes to their full potential (Fasoula 1990; Fasoula and Fasoula, 1995; 1997, 2000, 2002; Tokatlidis et al., 2006, 2010a, 2010b; Fasoula and Tokatlidis, 2012). The HC planting design has also allowed selection of single plants that are tolerant to abiotic and biotic stresses due to increased insect and pathogen pressure that allow exploitation of resistance in susceptible cultivars (Fasoula and Tokatlidis, 2012). Fasoula (2000) identified two cotton lines from a susceptible cultivar ‘Sindos 80’ that were tolerant to *Verticillium* wilt. In dry beans, Tokatlidis et al., 2010b selected two high yielding and stable lines from commercial cultivars that were tolerant to heat stress. Cultivars developed under the wide-spaced HC planting design have early emergence, have vigorous plants, and vigorous growth; thereby acquiring competitive ability against weeds, and withstand drought (Fasoula and Fasoula, 1997).

Pasini and Bos (1990) found that progenies of spring rye plants grown under low density were shorter, had an increased ear number per plant and increased yield per plot than progenies grown from high density spring rye. In the study, low and high density
plants were grown at inter-plant distances of 100 cm and 15 cm, respectively, in the first year, and progenies were evaluated in conventional rows under high density. Plant stand per plot was higher for progenies selected from plants grown at low density. This observation was attributed to increased seed quality as only better seeds were selected from the increased number of ears per plant at low density than from plants grown at high density.

Iliadis et al. (2003) evaluated three different interplant distances of 15 cm, 30 cm and 100 cm, to assess the effectiveness of selection variability in chickpea inbred lines. Selection variability was more effective at 30 cm followed by 100 cm interplant distances than at 15 cm. There was no visible variation among chickpea plants at 15 cm.

Genetic variation for multiple agronomic traits was reported in maize double haploid lines that were considered homozygous at every loci (Russell et al., 1963). The authors attributed this variation to mutations. Sprague and Penny (1963) detected allelic variation in maize inbred lines where expression of alleles was significantly different from the expected 1:1 ratio in 9 out of 15 genes.

Hopkins et al. (2013) identified unexpected wild type \( HTH \) progeny in F3 progenies of \textit{Arabidopsis hothead} \((hth)\) that were grown in complete isolation. The average wild type phenotypic expression in the F3 progeny was 1.53\%. In addition, the authors found one \textit{hth} mutant plant with shoot tissue that expressed both \textit{hth} and \textit{HTH} characteristics indicating variation within a single plant. This variation was attributed to cryptic variation present in previous generations that reappears in later generations due to stress (Lolle et al., 2005).
Other studies have attributed stress within-cultivar genetic variation to epigenetics or stress memory that may or may not persist in later generations. Kovalchuk et al. (2003) observed a 3-fold increase in recombination events in tobacco plants infected with a virus compared with the control plants regardless of the temperature or resistance levels to tobacco mosaic virus infection. The increase in recombination events indicated DNA rearrangement in response to the infection. Grafting a leaf from the infected plant onto other plants resulted in over a 2-fold increase in resistance compared with the control indicating heritability of the recombinant event. Molinier et al. (2006) observed 2- to 4-fold increases in homologous recombination frequencies (HRF) in Arabidopsis exposed to ultraviolet-C or bacterial infection. Further studies showed that the increase in recombination frequency could be expressed in progenies up to four generations without additional exposure to stress. Blodner et al. (2007) noted that progenies of Arabidopsis plants exposed to cold temperature at the bolting and seed maturing stages had higher photosynthetic rate when grown under chilling and freezing conditions. The higher photosynthetic rate was attributed to the up-regulation of flavanone 3-hydroxylase and pseudo response regulator 9 transcript, and down-regulation of transcription factors associated with growth. These changes in photosynthetic regulation resulted in better performance of the progeny. Pecinka et al. (2009) studied ten different abiotic stresses on Arabidopsis plants, and established that stress increased homologous recombination of somatic cells. Further studies with first- and second-generation Arabidopsis plants indicated a reduction in homologous recombination with each generation for the majority of the stresses, which was attributed to restoration to normal conditions after stress. The rate of restoration, however, depended upon the type of stress.
In canola, Hauben, et al. (2009) found lower and higher yielding plants from tissue culture isogenic double haploids than the control plants. These traits were stably passed on to progenies for eight subsequent generations in the greenhouse and for three generations in the field. These differences in performance were attributed to epigenetic changes as amplified fragment length polymorphism (AFLP) showed no genetic differences between tissue culture and the control plants.

Recent soybean studies identified aconitase isozymes allelic variation in homozygous cultivars exposed to environmental stimuli (Espinosa et al., 2015; Coleman et al., 2016). Aconitase (aconitate hydratase enzyme commission (EC) 4.2.1.3) catalyzes the reverse interconversion of citrate to isocitrate via a cis-aconitic acid intermediate in the Krebs cycle (Rouault and Klausner 1996; Sadka et al., 2000; Shlizerman et al., 2007). The Krebs cycle generates energy for organism’s metabolic processes (Schnarrenberger and Martin, 2002). Aconitase is comprised of iron-sulfur cluster (3Fe-4S) in its inactive form and is activated through the binding of a fourth free iron to form 4Fe-4S cluster (Zhou and Ragan 1995; Gardner 1997; Shlizerman et al., 2007). Cytosolic and mitochondrial forms of aconitase have been identified based on their location in the cell and have highly similar sequences (Doong and King, 1987; Peyret et al., 1995; Shlizerman et al., 2007). It is believed that mitochondrial aconitase operates in the Krebs cycles, while the cytosolic form is involved in a glyoxylate pathway (Hayashi et al., 1995; Peyret et al. 1995; Eprintsev et al., 2015). Five aconitase (Aco) isozymes, namely Aco1, Aco2, Aco3, Aco4 and Aco5, have been identified in soybeans (Griffin and Palmer, 1987; Kiang and Bult 1991; Terol et al., 2010; Espinosa et al., 2015). Studies with Aco isozyme in two soybean cultivars indicated allelic switching in one seed of a single,
three-seeded pod (Espinosa et al., 2015). Seed source for the experiment was from HC-grown, single-threshed plants that were exposed to natural hail, and a modified accelerated aging (AA) protocol before evaluation in conventional rows. Aconitase isozyme test on three-seeded pods collected from the conventional rows showed single and double allele switching at the Aco4 locus from Aco4-aa to Aco4-ab, Aco4-bb to Aco4-ab, or Aco4-aa to Aco4-bb in one cultivar, and Aco4-cc to Aco4-bc or Aco4-cc to Aco4-bb in the other cultivar. Self-pollination of Aco4-bc showed segregation in a 1bb:2bc:1cc ratio. Heritability studies with Aco4-bb and Aco4-cc variants indicated that the alleles were heritable and stable.

**Heat stress on agronomic performance and seed characteristics**

The accelerated aging exposes seeds to high temperature and humidity stresses. Rolling (2012) evaluated phenotypic variation in two soybean cultivars exposed to a modified AA at 41°C and 100% relative humidity for 48 h prior to planting in conventional rows. The modified AA treatment reduced plant stand, plant height and oil content but improved seed germination, vigor and yield in the stressed generation. High temperature stress also induces variation in agronomic performance, seed weight, seed quality and composition in soybean. Considerable final seed weight variation was noted in soybean cultivars grown under different temperatures. Seed weight on a dry matter basis was reduced by approximately 9% for every 3 °C increase in ambient temperature (Tacarindu et al., 2012). Studies by Dornbos and Mullen (1991) observed that high growth temperatures, especially at pod filling stage, caused significant variation in soybean seed yield, protein and oil contents compared with optimal growth temperature. When plants were exposed to increasing air temperatures from 29 to 34°C, yields were
reduced as a result of fewer pods per plant compared with the control. Similarly, variation was observed in soybean seed germination and vigor as a result of an increase in temperature (Egli et al., 2005). In the study, seed germination was reduced from 100% to 85% as temperature increased from 24 to 37°C and seed vigor was reduced to less than 11% during pod filling stage. These phenotypic observations could have a genetic basis, but these studies did not evaluate genetic variation.

**Defoliation stress on agronomic performance and seed characteristics**

The effect of hail damage stress on agronomic performance and seed characteristics of soybean has been simulated using plant defoliation. The effect depended on severity of the treatment, plant developmental stage and plant growth habit (Fehr et al., 1983). Studies have shown that soybean plants recover from defoliation by producing lateral branches if the treatment was applied during vegetative stages of plant development (Fehr et al., 1983; Wells, 1993). Fehr et al. (1981) determined the most critical stages of defoliation of determinate and indeterminate soybean varieties. The researchers applied 100% defoliation treatment at stages R4, R4.5, R5, R5.5 and R6. The most critical stages of defoliation were R5 or R5.5, which resulted in 80% average yield loss in both cultivars. In general, defoliated plants matured faster and were shorter when compared with the control plants (Fehr et al., 1981). These results concurred with earlier studies (Fehr et al., 1977) where defoliation at R5 and R5.5 resulted in an 85% average yield reduction. Gregorutti et al. (2012) studied the effect of soybean defoliation of 0, 33, 66 and 100% at plant developmental stages R3 and R5. Results from this study indicated that 66% and 100% plant defoliation levels imposed at R3 reduced yield by 27% and 90%, respectively, in comparison with the control treatment. Seed weight also was
reduced by 32% with 100% defoliation. Similar results were observed in guar, in which defoliation during the late growth period reduced yields and plant height (Sij et al., 2005). However, these studies also did not evaluate genetic changes in these individuals or their progenies.

Soybean plants exposed to multiple environmental stimuli produced seed variants with different phenotypic and genotypic characteristics (Espinosa et al., 2015). When these variants were selfed, the variation was heritable and stable. The seeds were produced in plants subjected to wide-spaced HC planting, natural hail and to a modified AA at planting. However, the effect of a single environmental stimulus on agronomic performance, seed characteristics and stability of variants from a soybean plant and its progeny had not been evaluated.

The objectives of this dissertation research were to evaluate (1) agronomic performance, seed characteristics and within-cultivar variation in progenies of two soybean cultivars, ‘BSR 101’ and ‘Jack’ grown in a wide-spaced HC planting design and conventional-row planting and exposed or not exposed to simulated hail (Chapter 2); (2) agronomic performance, seed characteristic and phenotypic variability in progeny from entries of ‘Jack’ variantb, ‘Jack’ variantc, and ‘Jack’ original cultivar planted in a wide-spaced HC planting design and treated with a modified AA (Chapter 3); and (3) the stability of the Aco4 isozymes in soybean seed from ‘Jack’ variants exposed to either a wide-spaced HC planting design or modified AA before planting and to determine genetic differences between variants and ‘Jack’ original foundation seed for Glyma.11g080600 (Chapter 4).
In objective 2, two independent experiments were conducted using (1) a wide-spaced HC planting design and (2) modified AA. In objective 3, aconitase isozyme profiles were evaluated and Aco4 gene, (Glyma.11g080600) was sequenced to determine DNA changes among samples.

**Dissertation organization**

The dissertation is organized into five chapters and includes three research papers. Chapter 1 is the general introduction. Chapter 2 focuses on the evaluation of agronomic performance, seed characteristics and within-cultivar variation in progenies of two soybean cultivars, ‘BSR 101’ and ‘Jack’, grown in a wide-spaced HC planting design and conventional-row planting and exposed or not exposed to simulated hail. Chapter 3 reports on the evaluation of agronomic performance, seed characteristics and phenotypic variability in progeny from entries of soybean cultivar ‘Jack’ variants planted in a wide-spaced HC planting design and treated with a modified AA. Chapter 4 focuses on the evaluation of stability of aconitase isozyme profiles in soybean ‘Jack’ variants from plants grown in a wide-spaced HC planting design and from seeds exposed to modified AA. Chapter 5 summarizes the general conclusions of this research.

**Reference**


CHAPTER 2. SOYBEAN AGRONOMIC PERFORMANCE AND SEED CHARACTERISTICS IN RESPONSE TO PLANT POPULATION AND SIMULATED HAIL

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Abstract

Soybean intra-cultivar genetic variation has been detected in inbred lines exposed to multiple environmental stimuli. However, the effect of a single environmental stimulus on intra-cultivar agronomic performance and seed characteristics variation in progeny of exposed soybean plants is not known. The objective of this study was to evaluate agronomic performance and seed characteristics in progeny from ‘BSR 101’ and ‘Jack’ soybean cultivars grown in a wide-spaced honeycomb (HC) planting design and conventional-row (CR) planting with and without simulated hail. Our results showed that progeny from ‘BSR 101’ plants grown in a wide-spaced HC planting design were vigorous, grew 8 cm taller, matured 3 d later and the 100-seed-weight was 0.5 g heavier than those from CR planting. The progeny from plants produced without simulated hail in a wide-spaced HC planting design and CR planting had 11% and 3% greater seed yield, respectively, than those with simulated hail. Protein content was 0.4% higher in progeny
from plants in CR planting with simulated hail than those without simulated hail. In ‘Jack’ progeny from plants in wide-spaced HC planting design, the 100-seed-weight was 0.23 g heavier than those in CR planting. Simulated hail treatment did not have a significant effect on agronomic performance or seed characteristics of the ‘Jack’ cultivar. These results show that intra-cultivar variation in highly inbred soybean lines exposed to different environmental stimuli can result in progeny with significant agronomic performance and seed characteristics variation. This information could be useful to plant breeders selecting new biotypes when breeding for new cultivars. It also could be important to soybean seed producers in explaining progeny performance variation when their seed production fields are affected by a hailstorm during the plant’s vegetative growth.

**Introduction**

Soybean plants, like any other living organisms, are exposed to a wide range of unfavorable environmental stimuli that may affect their performance. These conditions include plant density, high/low temperatures, UV-radiation, salt, drought, flooding, hail storm, nutrients and biotic stresses (Pencika et al., 2009; Tokatlidis et al., 2010; Krasensky and Jonak, 2012). Due to their immobility, plants develop mechanisms to adapt or respond to changing environments (Rasmusson and Phillips, 2010; Taiz and Zeiger, 2010). These mechanisms could be genetic, epigenetic, morphological, as well as physiological in nature (Walbot and Cullis, 1985; Molinier, 2006; Taiz and Zeiger, 2010; Boyko and Kovalchuk, 2011). Some changes may be beneficial and enhance plant adaptation to changing environments (Fasoula and Fasoula, 1997; Fasoula, 2000; Boyko
et al., 2010) and may create useful genetic variation in well-established cultivars (Rawlings et al., 1958; Fasoula and Boerma, 2005, 2007). This genetic variation may generate new individuals with enhanced agronomic performance, seed characteristics (Walbot and Cullis, 1985; Durrant, 1962), and other superior traits for use in plant breeding programs (Russell, et al., 1963; Fasoula et al., 2007a, 2007b, and 2007c).

Environmental stimuli also may enhance intra-cultivar variation in soybean (Byth and Weber, 1968; Fasoula and Boerma, 2005, 2007). The wide-spaced HC planting design is the most commonly studied environmental stimulus for generating intra-cultivar variation in plants. In this planting design, single plants are widely and equidistantly spaced throughout the field to reduce plant-to-plant competition (Fasoulas and Fasoula, 1995). Wide-spaced HC planting generates intra-cultivar genetic variability that could be used to develop new cultivars with superior characteristics from adapted cultivars, including inbred lines (Fasoula and Fasoula, 2002; Tokatlidis et al., 2004; Tokatlidis et al., 2006; Fasoula et al., 2007a, 2007b, and 2007c; Fasoula and Boerma, 2007; Tokatlidis, 2011).

Fasoula and Boerma (2007) uncovered intra-cultivar differences in seed protein and oil content for three soybean cultivars Benning, Cook, and Haskell. In their study, the average protein contents were 403 g kg$^{-1}$ DM in Benning, 435 g kg$^{-1}$ DM in Cook, and 394 g kg$^{-1}$ DM in Haskell; and the individual intra-cultivar protein content values ranged by 66 g kg$^{-1}$ DM in Benning, 64 g kg$^{-1}$ DM in Cook, and 88 g kg$^{-1}$ DM in Haskell. The average and range values for oil content were 217 and 37 g kg$^{-1}$ DM in Benning, 186 and 50 g kg$^{-1}$ DM in Cook, and 199 and 45 g kg$^{-1}$ DM in Haskell, respectively. Further studies on the three soybean cultivars resulted in selection and registration of a total of 19
soybean germplasm lines based on protein and oil content (Fasoula et al., 2007a, 2007b, and 2007c).

Plant population density affected yield in subsequent generations in two soybean populations. Tokatlidis et al. (2010) observed that soybean progeny selected under lower plant density in the field yielded 40% higher than those selected under higher density. These results showed that plants selected under lower density had a yield advantage over those planted at higher densities, possibly due to a genetic response in progeny seeds from parental plants to reduced inter-plant competition for available resources. Progenies from high yielding lines selected under low-density planting also yielded higher when planted under CR planting. However, a genotype by environment (GxE) interaction influenced performance of cultivars. This GxE interaction was evidenced by the different performance of the same lines when planted in the field and greenhouse.

Numerous studies evaluated the effect of hail and defoliation stress on agronomic performance and seed characteristics of the treated plants (Fehr et al., 1983; Hintz and Fehr 1990; Sij et al., 2005; Conley et al., 2008; Gregorutti et al. 2012; Moscardi et al., 2012). The level of variation in these studies depended on the intensity of the defoliation treatment, plant developmental stage, and plant growth habit. Defoliation during early stages of vegetative and reproductive development may have little effect on soybean performance due to the plants’ ability to compensate for reduced canopy or recover from defoliation by producing more lateral branches (Fehr et al., 1983; Sij et al., 2005; Moscardi et al., 2012).

However, the effect of a single environmental stimulus on intra-cultivar agronomic performance and seed characteristics variation in progeny of exposed soybean
plants is not known. The objective of this study was to evaluate agronomic performance, seed characteristics and intra-cultivar variation of progenies of two soybean cultivars, ‘BSR 101’ and ‘Jack’ from wide-spaced HC planting design and CR planting exposed or not exposed to simulated hail.

**Materials and methods**

**Plant material and seed source**

Two soybean cultivars ‘BSR 101’ (PI 548519) and ‘Jack’ (PI 540556) were used in the study. ‘BSR 101’ was released in 1985 by the Iowa Agriculture and Home Economics Experiment Station, USDA-ARS, and the Puerto Rican Agricultural Experiment Station. The cultivar is resistant to brown stem rot, caused by *Phialophora gregata* (Allington and Chamberlain) W. Gams, and has high yield (Tachibana et al., 1987). ‘Jack’ was released in 1989 by the Illinois Agricultural Experiment Station. It is resistant to Races 3 and 4 of soybean cyst nematode (SCN) caused by *Heterodera glycines* Ichinohe, and has high yield (Nickell et al., 1990). ‘BSR’ 101 and ‘Jack’ belong to maturity groups I and II, respectively (Tachibana et al., 1987; Nickell et al., 1990).

Seed from 200 single-threshed plants of ‘BSR 101’ and ‘Jack’ were obtained from Drs. R.G. Palmer and R. Shoemaker, USDA ARS-Iowa State University, Ames, IA, respectively. Each single plant was assigned a number called ‘entry’ from 1-200. For all experiments, plant developmental stages are defined according to Fehr and Caviness (1971).
**Wide-spaced HC and CR planting designs**

In 2011, 50 entries of each cultivar was planted in a wide-spaced HC planting design as described by Fasoulas and Fasoula (1995) where hills were 2.8 m apart from each other in all directions. Four seeds were planted per hill and seedlings were thinned to one healthy plant at V2-V3 stage of development. Two repetitions (hills) per entry were planted. Plants from one hill were exposed to a simulated hail treatment and plants from the second hill were allowed to grow undisturbed and used as controls. Additionally, seeds from the same 50 entries of each cultivar were planted in CR. Two plots of each entry were planted in single-row plots of 3.5 by 0.76 m. The first plot was used for the simulated hail treatment and the second plot was used as a control (Fig. 3).

**Simulated hail treatment**

Simulated hail treatment was imposed at V4-V5 soybean growth stage in 2011. Two thirds (66 %) of the trifoliate leaves were removed from every single wide-spaced HC and CR plants with a pair of scissors (Fig. 2). The treatment was applied to all plants on the same day. The study had a total of four treatments per cultivar: (i) 50 entries planted in a wide-spaced HC planting design with simulated hail; (ii) 50 entries planted in wide-spaced HC planting design without simulated hail (control); (iii) 50 entries in a CR planting with simulated hail; and (iv) 50 entries in CR planting without simulated hail (control).

At maturity, each entry was single-plant or row-plant threshed and identified with the entry number. Seeds were increased in 2012 and 2013 at Iowa State University, Bruner Research Farm, near Ames, IA to produce sufficient seed for agronomic performance experiments in 2013 and 2014, respectively. Seed from each entry and
treatment were multiplied by planting in two-row plots of 3-m in length, and combine harvested at maturity.

**Location**

The experiment was conducted at three locations: Iowa State University, Sorenson Research Farm, near Ames, IA (42° 0’ 47” N, 93°44’40” W) on a Canisteo clay loam (Webster clay loam) soil in 2013; Worle Farm near Ames IA (41° 59’ 54” N, 93°41’43” W) on a Clarion loam (Canisteo silty clay loam) in 2014; and Glidden, IA (42° 3’ 54” N, 94° 37’ 22” W) on a Nicollet loam soil in 2014. Two hundred and eighty seeds of each entry were planted in two-row plots of 5.3 m long and 0.76 m between rows in all locations.

The experiment was designed as a factorial arrangement in a randomized complete block design blocked by location-year (Ames 2013, Ames 2014 and Glidden 2014). The two cultivars were planted in separate blocks side by side. Planting pattern, (wide-spaced HC planting design and CR planting), was considered a main plot factor, and simulated hail treatment, (simulated and non-simulated hail), was considered a sub-plot factor.

**Data collection**

Data collected on each entry included plant stand, days to maturity, plant height, seed yield, 100 seed weight, standard seed germination, seed vigor, seed oil and protein content.

**Plant stand:** All healthy emerged seedlings were counted at three weeks after emergence.
Days to maturity: Harvest maturity was determined when at least 95% of the pods attained a mature brown color and data were expressed as number of days from September 1.

Plant height: Plant height (cm), was measured from three randomly selected plants within each plot. Each plant was measured from the soil surface to the terminal node of the plant at harvest maturity (Fehr et al., 1977). The three plant heights were averaged to obtain plant height per entry.

Seed yield: Seed yield per plot was determined at harvest with an ALMACO 40 plot combine (ALMACO, IA, USA). Seed yield was recorded in kg ha\(^{-1}\), after standardizing to 13 % moisture content.

Seed weight: One-hundred-seed weight (g) was calculated by averaging weight of two sub-samples of 100-seeds per entry.

Standard seed germination: Seed germination was conducted according to the Association of Official Seed Analysts (AOSA) Rules for Seed Testing (2013). One hundred seeds per entry were planted on top of crepe cellulose paper (Kimberly Clark, Neenah, WI) moistened with 840 ml of water on a fiberglass tray (45 cm x 66 cm x 2.54 cm). The tray with seed was incubated at 25 °C with 8 h of light and 16 h of darkness day\(^{-1}\). Seedling evaluation was done 7 d after planting. Seedlings were categorized into normal, abnormal, hard and dead seeds (AOSA, 2013). Values were expressed in %.

Seed vigor test: Seed vigor was determined using the accelerated aging (AA) test according to AOSA Seed Vigor Testing Handbook (AOSA, 2009) procedure. One hundred seeds per entry were placed in a single layer on a wire mesh inside a 10 x 10 x 4 cm plastic AA box containing 40 ml of distilled water. The boxes were incubated in an
AA chamber (VWR Scientific Inc.) at 41 °C and 100 % relative humidity for 72 h. After AA, the seeds were planted on moist crepe cellulose paper on a fiberglass tray. Four entries were planted per tray. The samples were covered with moist sand, and incubated at 25 °C with 8 h of light and 16 h of darkness day\(^{-1}\). The percentage of normal seedlings in the AA test was evaluated 7 d after planting (AOSA, 2013).

**Seed composition:** Seed oil and protein content expressed in percentage was measured with near-infrared transmittance (NIT) using an Infratec 1229 Grain Analyzer (Foss Tecator AB, Hoganas, Sweden).

**Data analysis**

Analysis of variance was performed using JMP Statistical Software (JMP Pro 12.0.1, SAS Institute Inc., 2015) to analyze treatment effects on agronomic performance and seed characteristics. The dataset was tested for the assumptions of normality and homogeneity of variances. No data transformation was required for all traits. Mean estimates were calculated, and Student’s t-test was used to determine the significant difference in responses among main effects. Where interactions were significant, contrasts were used to determine the difference between levels of one factor at each level of another factor. In this study, blocks (location-years) were considered random, while planting pattern and simulated hail treatment were considered fixed factors. Each cultivar was analyzed separately as the two cultivars were grown in different blocks and belong to two different maturity groups. All tests were done at \( P \leq 0.05 \) level.
Results

Results from the study showed no interaction between planting pattern and simulated hail treatment for all agronomic performance and seed characteristics evaluated, except for seed yield and percent protein content in progeny plants of ‘BSR 101’. Simulated hail treatment had no effect on agronomic performance and seed characteristics measured in both cultivars.

Significant differences were found between progenies from plants in wide-spaced HC planting design and CR planting for days to maturity, plant height, and 100-seed weight in ‘BSR 101’, and only for 100-seed weight in ‘Jack’ cultivar. The difference between progenies from plants in a wide-spaced HC planting design and those in CR planting for the rest of the characteristics were not significant.

For ‘BSR 101’, progenies from plants in wide-spaced HC planting design matured 3 d later \((P < 0.05)\) than progenies from plants in CR planting. Progenies from plants in a wide-spaced HC planting design were 8 cm taller \((P < 0.05)\) than those in a CR planting. One hundred-seed weight was significantly heavier in progenies from plants in a wide-spaced HC planting design by 0.5 g \((P < 0.05)\) compared with progenies from plants in a CR planting (Table 1).

Differences between progenies from plants in a wide-spaced HC design and CR were not significantly different for standard seed germination, seed vigor and seed oil content. However, progenies from plants in a wide-spaced HC planting design had 5 % and 3 % higher standard seed germination and seed vigor, respectively, compared with progenies from plants in a CR planting (Table 1).
There was significant interaction between planting pattern and simulated hail treatment for seed yield and protein content in ‘BSR 101’ (Table 2). Due to this interaction, the effect of planting pattern for each level of simulated hail treatment was statistically tested for both yield and protein content. Results indicated that progenies from plants in a wide-spaced HC planting design without simulated hail yielded 11% higher ($P < 0.01$) compared with progenies from plants in a wide-spaced HC planting design with simulated hail. There was a 3% seed yield increase ($P < 0.05$) in progenies from plants in a CR planting without simulated hail than those with simulated hail. The progenies from plants in a CR planting with simulated hail had 0.4% higher protein ($P < 0.01$) when compared to progenies from plants in CR planting without simulated hail. No significant differences were found for protein content in progenies from plants in a wide-spaced HC planting design with and without simulated hail treatment.

There were no significant differences for number of days to plant stand, maturity, plant height, 100-seed weight, standard seed germination, seed vigor and seed oil between progenies from plants in non-simulated and simulated hail treatments (Table 3).

For ‘Jack’ cultivar, there were no significant differences between progenies from plants in a wide-spaced HC planting design and those in CR planting for plant stand, maturity, seed yield, standard germination, seed vigor, oil and protein content except for 100-seed weight (Table 4). Progeny seed from plants in wide-spaced HC planting design were 0.23 g ($P < 0.05$) heavier than progeny seed from plants in a CR planting. No significant differences were found between progenies from plants in a non-simulated and simulated hail treatment for all agronomic performance and seed characteristics in ‘Jack’ cultivar (Table 5).
Intra-cultivar variation was evaluated in progenies from plants grown in a wide-spaced HC planting design and those in a CR planting. No significant intra-cultivar variation was found in ‘BSR 101’ and ‘Jack’ progenies.

**Discussion**

The study was designed to evaluate agronomic performance and seed characteristics, and intra-cultivar variation in progenies of BSR 101’ and ‘Jack’ soybean cultivars from wide-spaced HC planting design and CR planting with and without simulated hail. Simulated hail treatment did not modify agronomic performance in both cultivars but wide-spaced HC planting design did.

Other authors have reported enhanced intra-cultivar variation expressed in soybean as a consequence of using a wide-spaced HC planting design (Fasoula and Boerma, 2005, 2007; Fasoula et al., 2007a, 2007b, 2007c). These authors reported that a wide-spaced HC planting design eliminated interplant competition and allowed full expression of intra-cultivar variation. When single plants selected from a wide-spaced HC planting design were evaluated under optimal density, they had improved performance compared with those plants selected under higher density planting (Kyriakou and Fasoula, 1985; Fasoula, 1990). Prior research by our research team showed that intra-cultivar variation and new biotypes existed in plants exposed to natural hail in a wide-spaced HC planting design (Espinosa et al., 2015; Sandhu et al., 2016). Our current study, for the first time, investigates and quantifies the effect of planting design separate from that of hail.
The progeny from ‘BSR 101’ wide-spaced HC planting design matured later than the progeny from CR planting. This difference could be due to vigorous plant growth in wide-spaced HC planting design progenies that resulted in bigger and taller plants (Cober and Morrison, 2010; Kato et al., 2015). A strong positive relationship between plant height and maturity was reported in previous studies (Anand and Torrie, 1963; Lin and Nelson, 1988). In our study, the relationship between plant height and maturity was positive although not strong (data not shown). Vigorous plant growth also could be the result of better seed vigor from progenies from wide-spaced, HC planting design plants that resulted in early emergence, vigorous shoot and extensive root system, and vigorous growth thereby acquiring competitive ability against stresses (Fasoula and Fasoula, 1997; Rahman et al., 2005; Fasoula and Tokatlidis, 2012). In our study, progenies from plants grown in a wide-spaced HC planting design were significantly taller than those from CR planting (Table 1). The abundance of sunlight and nutrients available to single plants in a wide-spaced HC planting design might stimulate production of vigorous seed, promote expression of intra-cultivar variation and enhance production of new phenotypes in the progeny.

Mean 100-seed weight was consistently and significantly higher in progenies from plants grown in a wide-spaced HC planting design than those from CR planting in both ‘BSR 101’ and ‘Jack’ cultivars. This increase in seed weight could be the result of improved vegetative plant growth as evidenced by increased plant height (Fig. 1A) that lengthened seed-fill duration (Egli, 2004). Progenies from plants in a wide-spaced HC planting design were more vigorous and matured later compared to progenies from the CR planting. This vigorous plant growth and prolonged growth period could contribute
significantly to seed dry matter accumulation resulting in larger seeds (Mansur et al., 1996; Cicek et al., 2005; Moles et al., 2006; Nik et al., 2011; Ali et al., 2013; Kawasaki et al., 2016). A significantly positive relationship between seed filling period and seed size/weight was reported in three soybean cultivars (El-Zeadani et al., 2014). Although these authors did not report a significant relationship between seed filling period and planting density, in their study, bigger seed were observed from soybean plants grown under lower density than under higher density. Other studies also have reported larger seed size and other traits in progenies of entries selected from wide-spaced HC planting design, and evaluated in progeny CR (Fasoula et al., 2007a, 2007b, and 2007c). Perhaps seed weight would be an effective trait to use in breeding programs to identify intra-cultivar genetic variation in progenies.

The interaction between planting pattern and simulated hail treatment for seed yield and protein content was significant in ‘BSR 101’ (Table 2). Progenies from plants grown in both a wide-spaced HC planting design and CR planting without simulated hail yielded significantly greater than those from simulated hail, which was expected since hail reduced plant biomass. Other authors have reported a positive relationship between plant biomass and availability of photosynthetic products and seed yield (Vieira et al., 1992; Borrás et al., 2004; Gregorutti et al., 2012). In our study, yields per ha were significantly higher in progenies from plants grown in a wide-spaced HC planting design not exposed to hail. These plants were more vigorous than those in CR planting, had a longer seed-fill period, and increased seed weight (Anand and Torrie, 1963; Wilcox and Sediyama, 1981; Cui and Yu, 2005; Arshad et al., 2006; Malik et al., 2007; Ali et al., 2013). Plant height had a positive relationship with yield in our study until reaching a
plateau before yield decreased with increasing plant height (Fig. 1B). This observation indicated that yield was also a function of other factors such as seed weight, number of seed per plant in addition to plant height (Wilcox and Sediyama, 1981; Meckel et al., 1984; Berger-Doyle et al., 2014). Wilcox and Sediyama, (1981) reported a 350 kg ha\(^{-1}\) and 112 kg ha\(^{-1}\) seed yield increase in determinate- and indeterminate-growth soybean cultivars, respectively, for every 10 cm increase in plant height. A positive relationship between seed yield and seed weight in soybean was reported in previous studies (Meckel et al., 1984; Specht et.al., 1986; Fasoula and Boerma, 2007).

Protein content was higher in progenies from plants grown in CRs with simulated hail. These progenies had lower seed yield than progenies without simulated hail. These results corroborated findings from studies in chickpea where seed yield and protein content also were negatively correlated (Wilcox and Cavins, 1995; Frimpong et al., 2009; Gangola et al., 2012; Gaur et al., 2016). Gaur et al. (2016) reported reduced protein content in chickpea when seed size and seed yield increased, which they attributed to changes in starch/protein ratio. In our study, seed yield increased while protein content remained unchanged in progenies from plants grown in a wide-spaced HC planting design with and without simulated hail.

Plant breeders selecting for both traits could use a wide-spaced HC planting design to uncover superior characteristic (Fasoula and Boerma, 2007), thereby increasing selection efficiency (Fasoula and Fasoula, 1995). Additionally, Wilcox and Cavins (1995) showed that superior seed yield and higher protein content cultivars may be obtained through backcrossing a high seed protein soybean cultivar to a high yielding cultivar. These results suggest that high yielding lines could be selected from our study
and backcrossed to high protein content lines to produce high yielding and high protein content cultivars.

The agronomic performance of progenies from plants subjected to simulated hail was not different from those of non-simulated hail plants. This lack of response to hail stress could be the result of soybean plants’ vegetative growth-habit. Soybean plants have the ability to recover from a reduced plant stand or damage by producing lateral branches when defoliation happens early during vegetative growth (Fehr et al., 1983; Wells, 1993; Vazquez et al., 2008; Moscardi, 2012). In our study, we applied 66% reduction in leaf area at V4-V5 stage, which did not affect agronomic performance of progenies from ‘BSR 101’ and ‘Jack’ cultivars.

**Conclusion**

The agronomic performance and seed characteristics of progenies from plants planted in a wide-spaced HC planting design were significantly better for most traits evaluated than progenies from CR planting. Simulated hail treatment did not have a significant effect on agronomic performance and seed characteristics. Prior research showed agronomic variation and new biotypes (Espinosa et al., 2015; Sandhu et al., 2016) in progeny from plants in a wide-spaced HC planting design exposed to hail. Our study, for the first time, separates the environmental effects of planting distance and simulated hail on the agronomic performance, seed characteristics, and intra-cultivar variation in progeny of the affected plants. These results show that intra-cultivar variation in homozygous lines exposed to different environmental stimuli can result in progeny with significant agronomic performance variation. This information could be
useful to plant breeders when using these tools to select new biotypes. It also could be important to soybean seed producers in explaining progeny performance variation when their production fields are affected by a hailstorm during the plant’s vegetative growth. Future studies should assess the expression and stability of superior traits in progeny from wide-spaced HC planting design for multiple generations.

**Acknowledgements**

We would like to thank the Agronomy Department, Seed Science Center and American Seed Trade Association for partial funding of this research. Our appreciation is expressed to Iowa Crop Improvement Association for planting and managing all progeny plots from where the reported data was collected. Special thanks is extended to everyone who helped with data collection.

**References**


Tables and Figures

Table 1. Means of planting pattern (wide-spaced honeycomb planting design and CR planting) for days to maturity, plant height, 100-seed weight, standard germination, seed vigor, and oil content in ‘BSR 101’ across simulated hail treatment.

<table>
<thead>
<tr>
<th>Trait</th>
<th>HC design</th>
<th>CR planting</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maturity (days from Sept 1)</td>
<td>26.0 a†</td>
<td>23.00 b</td>
<td>±0.25</td>
</tr>
<tr>
<td>Plant Height (Cm)</td>
<td>84.75 a</td>
<td>77.74 b</td>
<td>±0.81</td>
</tr>
<tr>
<td>Seed weight (g 100⁻¹)</td>
<td>17.31 a</td>
<td>16.81 b</td>
<td>±0.05</td>
</tr>
<tr>
<td>Standard germination (%)</td>
<td>88.00</td>
<td>83.00</td>
<td>±0.98</td>
</tr>
<tr>
<td>Seed vigor (%)</td>
<td>84.00</td>
<td>81.00</td>
<td>±1.33</td>
</tr>
<tr>
<td>Oil content (%)</td>
<td>19.09</td>
<td>19.08</td>
<td>±0.03</td>
</tr>
</tbody>
</table>

† Means followed by different letters within a row are significantly different at $P < 0.05$. SE means standard error.

Table 2. Means of the interaction effect between planting pattern and simulated hail treatment for seed yield and protein content in progenies of ‘BSR 101’.

<table>
<thead>
<tr>
<th>Planting pattern</th>
<th>Hail Treatment</th>
<th>Yield (Kg ha⁻¹)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>NS</td>
<td>2933.15 a†</td>
<td>34.71 a</td>
</tr>
<tr>
<td></td>
<td>SH</td>
<td>2606.47 b</td>
<td>34.73 a</td>
</tr>
<tr>
<td>CR</td>
<td>NS</td>
<td>2662.37 a</td>
<td>34.68 b</td>
</tr>
<tr>
<td></td>
<td>SH</td>
<td>2586.58 b</td>
<td>34.82 a</td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td>±9.26</td>
<td>±0.004</td>
</tr>
</tbody>
</table>

† Means followed by different letters within a column for each planting pattern and variable is significantly different at $P < 0.05$. NS means no simulated hail. SH means simulated hail.
Table 3. Means for plant stand, maturity, plant height, 100-seed weight, standard germination, seed vigor, seed oil and seed protein content in ‘BSR 101’ progenies from non-simulated and simulated hail across planting patterns

<table>
<thead>
<tr>
<th>Trait</th>
<th>Non-Simulated hail</th>
<th>Simulated hail</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant Stand (No of plants emerged)</td>
<td>202.01</td>
<td>209.85</td>
<td>±1.52</td>
</tr>
<tr>
<td>Maturity (days from Sept 1)</td>
<td>24.00</td>
<td>24.00</td>
<td>±0.25</td>
</tr>
<tr>
<td>Plant Height (Cm)</td>
<td>83.36</td>
<td>79.13</td>
<td>±0.81</td>
</tr>
<tr>
<td>Seed weight (g 100⁻¹)</td>
<td>17.06</td>
<td>17.07</td>
<td>±0.05</td>
</tr>
<tr>
<td>Standard germination (%)</td>
<td>86.00</td>
<td>86.00</td>
<td>±0.98</td>
</tr>
<tr>
<td>Seed vigor (%)</td>
<td>83.00</td>
<td>83.00</td>
<td>±1.33</td>
</tr>
<tr>
<td>Seed Oil (%)</td>
<td>19.10</td>
<td>19.07</td>
<td>±0.03</td>
</tr>
</tbody>
</table>

Table 4. Means for plant stand, maturity, plant height, 100-seed weight, standard germination, seed vigor seed oil and protein content in progenies of ‘Jack’ cultivar from HC planting design and CR planting across simulated hail treatment

<table>
<thead>
<tr>
<th>Trait</th>
<th>HC design</th>
<th>CR planting</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant Stand (No of plants emerged)</td>
<td>192.32</td>
<td>199.41</td>
<td>±4.12</td>
</tr>
<tr>
<td>Maturity (Days from Sept 1)</td>
<td>34.00</td>
<td>33.00</td>
<td>±0.30</td>
</tr>
<tr>
<td>Plant height (Cm)</td>
<td>108.84</td>
<td>104.80</td>
<td>±0.83</td>
</tr>
<tr>
<td>Yield (Kg ha⁻¹)</td>
<td>2928.49</td>
<td>2877.72</td>
<td>±42.40</td>
</tr>
<tr>
<td>Seed weight (g 100⁻¹)</td>
<td>16.16 a†</td>
<td>15.93 b</td>
<td>±0.04</td>
</tr>
<tr>
<td>Standard germination (%)</td>
<td>94.00</td>
<td>94.00</td>
<td>±0.36</td>
</tr>
<tr>
<td>Seed vigor (%)</td>
<td>91.00</td>
<td>92.00</td>
<td>±0.29</td>
</tr>
<tr>
<td>Seed oil (%)</td>
<td>18.60</td>
<td>18.61</td>
<td>±0.03</td>
</tr>
<tr>
<td>Seed Protein (%)</td>
<td>35.52</td>
<td>35.49</td>
<td>±0.04</td>
</tr>
</tbody>
</table>

†Means followed by different letters within a row are significantly different at \( P < 0.05 \)
Table 5. Means for plant stand, maturity, plant height, seed yield, 100-seed weight, standard germination, seed vigor seed oil and protein content in progenies of ‘Jack’ cultivar from non-simulated hail and simulated hail across planting pattern

<table>
<thead>
<tr>
<th>Trait</th>
<th>Non-simulated Hail</th>
<th>Simulated Hail</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant Stand (No of plants emerged)</td>
<td>194.06</td>
<td>197.68</td>
<td>±4.20</td>
</tr>
<tr>
<td>Maturity (Days from Sept 1)</td>
<td>33.00</td>
<td>33.00</td>
<td>±0.30</td>
</tr>
<tr>
<td>Plant height (Cm)</td>
<td>107.95</td>
<td>105.69</td>
<td>±0.83</td>
</tr>
<tr>
<td>Yield (Kg ha(^{-1}))</td>
<td>2979.00</td>
<td>2826.22</td>
<td>±42.40</td>
</tr>
<tr>
<td>Seed weight (g 100(^{-1}))</td>
<td>16.00</td>
<td>16.09</td>
<td>±0.04</td>
</tr>
<tr>
<td>Standard germination (%)</td>
<td>94.00</td>
<td>94.00</td>
<td>±0.36</td>
</tr>
<tr>
<td>Seed vigor (%)</td>
<td>91.00</td>
<td>91.00</td>
<td>±0.29</td>
</tr>
<tr>
<td>Seed oil (%)</td>
<td>18.58</td>
<td>18.64</td>
<td>±0.03</td>
</tr>
<tr>
<td>Seed Protein (%)</td>
<td>35.52</td>
<td>35.49</td>
<td>±0.04</td>
</tr>
</tbody>
</table>
Figure 1. Relationship between (A) plant height and 100-seed weight, (B) plant height and yield in progenies of ‘BSR 101’ from plants in wide-spaced HC design across simulated hail treatment.
Figure 2. Simulated hail treatment imposed on ‘BSR 101’ and ‘Jack’ cultivars at V4-V5 stage in 2011 planted in a wide-spaced HC planting design and CR. (Photo by Dr Katherine Espinosa, 2011)
Figure 3. Seed Source for 2013 and 2014 agronomic performance and seed characteristics study.
CHAPTER 3. AGRONOMIC PERFORMANCE AND SEED CHARACTERISTICS IN ‘JACK’ VARIANTS EXPOSED TO ENVIRONMENTAL STIMULI

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Agronomy Department, Iowa State University, Ames, IA 50011-1010, USA.

Abstract

Soybean from ‘Jack’ cultivar subjected to environmental stimuli of wide-spaced honeycomb (HC) planting design and modified accelerated aging (AA) generated new biotypes. These new biotypes had different phenotypic characteristics and when selfed, produced homozygous variants for the new allele. However, the effect of environmental stimuli on agronomic performance and seed characteristic of these new biotypes was unknown. The objective of this study was to evaluate agronomic performance, seed characteristics and phenotypic variability in progeny from entries of ’Jack’ variant\(^b\), ’Jack’ variant\(^c\), and ‘Jack’ original cultivar planted in a wide-spaced HC planting design and those from plants whose seeds were treated with a modified AA at planting. Our results showed that agronomic performance and seed characteristics of progeny from entries within ’Jack’ variant\(^b\) and ’Jack’ variant\(^c\) were more variable than those in Jack’ original cultivar almost for all traits evaluated. For instance, in progeny from entries in wide-spaced HC planting design, the range in maturity was 7 d in ’Jack’ variant\(^b\) and 14 d in ’Jack’ variant\(^c\), while all entries matured at the same time in ‘Jack’ original cultivar. The ranges in 100-seed weight were approximately 1 and 2 g in ’Jack’ variant\(^b\) and ’Jack’
variant\textsuperscript{c}, respectively, but no variation in 100-seed weight occurred in ‘Jack’ original cultivar. In progenies from entries whose seeds were treated with modified AA, the range in maturity was 3 d in ‘Jack’ original cultivar, 4 d in ‘Jack’ variant\textsuperscript{b} and 9 d in ‘Jack’ variant\textsuperscript{c}. The range in 100-seed weight was approximately, 2g in both ‘Jack’ variant\textsuperscript{b} and ‘Jack’ variant\textsuperscript{c}, but there were no significant differences in the ‘Jack’ original cultivar. Variants had higher yield, oil content, shorter or longer maturity, and differed in other agronomic and seed characteristics. Agronomic performance differences in progenies of cultivars exposed to environmental stimuli can be substantial and could be useful to plant breeders when breeding for new cultivars with improved agronomic performance and seed characteristics.

**Introduction**

Environmentally induced phenotypic variation can be expressed in nearly-homozygous cultivars of soybean (Durrant, 1962; Walbot and Cullis, 1985; Stephens et al., 1991; Fasoula and Boerma 2005, 2007; Yates, et al., 2012). New cultivars derived from single-plants selected from a wide-spaced HC planting design have performed significantly better than the original cultivar (Fasoula and Fasoula 2000; Fasoula and Boerma 2005), and were selected in new cultivar development (Stephens et al., 1991; Fasoula, 2000; Fasoula and Boerma, 2007; Fasoula, 2012). The sources of this phenotypic variation may be attributed to residual heterozygosity, de novo variation or epigenetic responses that can occur as a genome-wide response to environmental stimuli (McClintock, 1984; Fasoula and Fasoula, 2000; Fasoula and Boerma, 2007; Haun et al., 2011; Yates et al., 2012). Fasoula and Boerma (2005; 2007) have released new soybean
lines selected from cultivars Benning, Cook and Haskell grown under ultra-low plant density. These single-plant descendants were evaluated in conventional row planting and had different plant height, maturity, seed weight, oil and protein content than the original cultivars. The authors found new soybean lines with higher and lower oil and protein contents, earlier and later maturing plants, uniformly taller and shorter plants, heavier or lighter seed weights and higher yielding plants than the original parents. These breeders later released a total of 19 new soybean lines varying in oil and protein contents (Fasoula et al., 2007a, 2007b, and 2007c).

Another environmental stimulus used to induce within cultivar variation is a modified accelerated aging (AA) test protocol (Rolling, 2012; Espinosa et al., 2015). AA is one of the most widely used seed vigor tests for soybean, providing useful information on seed storage potential and field emergence capability under sub-optimal environments (McDonald, 1999; Hsu, et al., 2003; Ghassemi-Golezani et al., 2010; Marcos-Filho, 2015). In this test, seeds are exposed to 41°C and 100% relative humidity for 72 h before planting (AOSA 2009). AA deteriorates seeds and accelerates the metabolic changes involved in seed aging. These metabolic changes are increased lipid peroxidation, reduced seed antioxidants, impaired activity of free radical-scavenging enzymes, and damaged nucleic acids and proteins (Parrish and Leopold, 1978; Chiu et al., 1995; Hsu and Sung, 1997; Bailly et al., 1998; McDonald, 1999; Hsu et al., 2003; Ghassemi-Golezani et al., 2010; Kumar, et al., 2015). Seed aging also negatively affects seed vigor and thus, hinders seed agronomic performance under a wide range of environmental conditions (Rajala et al., 2011; Ghassemi-Golezani et al., 2008; Ghassemi-Golezani et al., 2011; Ghahfarokhi et al., 2014). Rolling (2012) evaluated the effect of a modified AA on
within-cultivar variation in soybean. The artificially-aged seeds at planting time from two soybeans cultivars (‘Jack’ and ‘BSR-101’) had reduced plant stand and plant height. These seeds produced plants that had greater within-cultivar variation for agronomic performance and seed characteristics that were different than those from the control treatment. Espinosa et al. (2015) found unexpected allelic variation in progeny from plants exposed to environmental stimuli. The authors reported a single and, in some cases, a double allele-switch at the aconitase-4 (\(Aco4\)) locus for one seed within a 3-seeded pod. These new biotypes were considered variants from the ‘Jack’ original cultivar. These variants could be used as a source of new alleles in a breeding program.

However, the effect and extent of environmental stimuli on agronomic performance and seed characteristics of these new biotypes were unknown. The objective of this study was to evaluate agronomic performance, seed characteristics and phenotypic variability in progenies from entries of 'Jack' variant\(^b\), 'Jack' variant\(^c\), and 'Jack' original cultivar planted in a wide-spaced HC planting design and treated with a modified AA.

**Materials and Methods**

**Plant material and seed source**

Seed for this study was obtained from an experiment initiated in 2008 (Espinosa et al., 2015) and was summarized in Fig. 1. The original Espinosa et al. (2015) experiment was initiated in 2008 using seed obtained from Dr. Randy Shoemaker, USDA ARS-Iowa State University, Ames, IA (Fig. 1). In this original study, five cultivars of soybean, including 'Jack', were grown in a wide-spaced HC planting design. During the growing season, plants were severely damaged by a hail storm, and yield in
most plants was reduced. However, seed from a few surviving plants were single-plant threshed and assigned an ID number. In 2009, 64 of these entries from ‘Jack’ cultivars were stressed using a modified AA. The modified AA treatment consisted of exposing soybean seeds to 41°C temperature, and 100% relative humidity for 48 h, instead of the recommended 72 h (AOSA, 2009) before field planting. Two replications per entry were planted in conventional rows. At harvest maturity three, 3-seeded pods were collected from three randomly selected plants per cultivar, entry, and replication. The aconitase isozyme test was conducted on every seed from all pods using starch-gel electrophoresis for a total of 1152 seeds per cultivar. Results from the test indicated that a single-allele switch had occurred from the usual $Aco4$-$cc$ to $Aco4$-$bc$, in one seed of a 3-seeded pod collected from ‘Jack’ entry 127 replicate 1, in addition to four seeds that had double allele-switching from $Aco4$-$cc$ to $Aco4$-$bb$ (Espinosa et al., 2015). The $Aco4$-$bc$ plant was self-pollinated and alleles segregated in a normal $1bb:2bc:1cc$ ratio. Stability studies with $Aco4$-$bb$ and $Aco4$-$cc$ variants, denoted in this study as ’Jack’ variant$^b$ and ’Jack’ variant$^c$, indicated that the alleles were heritable and stable (Espinosa, et al., 2015). In 2011, 50 plants per variant were single plant-threshed and each plant was assigned an entry number. In 2012, seed from the 50 entries per variant were increased for subsequent studies.

Our study was initiated in 2013 and used three genotypes from Espinosa et al. (2015). Three entries per variant (Variants $Aco4$-$bb$ and $Aco4$-$cc$) and three other entries from ‘Jack’ original cultivar with $Aco4$-$cc$ from Espinosa et al. (2015) were used in this study to evaluate stability of the $Aco4$ isozyme alleles when exposed to additional stimuli (Fig. 1). The genotypes were: ‘Jack’ original cultivar, ’Jack’ variant$^b$ and ’Jack’
variant\textsuperscript{c}. Genotypes were named as follows: cultivar and type (original or variant) and super-script b and c indicated allelic patterns bb or cc. Each genotype had three entries according to the 2008 experiment nomenclature (Espinosa et al., 2015). The nomenclature used to identify the entries in this study were: ‘A’ indicated aconitase gene; entry number indicated plant number in the original experiment; type was indicated by a superscript, no superscript beside the plant number indicated ‘Jack’ original type and a superscript (v) indicated a variant; and bb or cc indicated the allelic pattern. The entries of ‘Jack’ variant\textsuperscript{b} included in this study were A8\textsuperscript{v}-bb, A13\textsuperscript{v}-bb and A41\textsuperscript{v}-bb; the entries of ‘Jack’ variant\textsuperscript{c} were A9\textsuperscript{v}-cc, A31\textsuperscript{v}-cc and A40\textsuperscript{v}-cc; and the entries of the ‘Jack’ original cultivar were A58-cc, A163-cc and A182-cc. Two separate experiments were conducted to evaluate the stability of these variants: a wide-spaced HC planting design and a modified AA.

**Wide-spaced HC planting design**

In 2013, a total of 25 seeds of each of the nine entries (Fig. 1) were wide-space planted in a HC pattern at a plant-to-plant spacing of 2.8 m, for a total of 225 plants. These plants were assigned plant numbers from 37-261. For instance, the nomenclature used to identify the entries and plants in this study were A13\textsuperscript{v}-bb-41, where ‘A’ indicated the aconitase gene; ‘13’ indicated the entry number from the original 2008 experiment; genotype was indicated beside the 2008 entry number by a superscript (\textsuperscript{v}) for variant or no superscript for the ‘Jack’ original cultivar; bb or cc indicated the allelic pattern; and the new 2013 entry number was indicated by plant number, ‘41’. Four seeds were planted per hill, and thinned to one healthy plant at V2-V3.
Modified AA

In 2013, 50 seeds from each of the nine entries were exposed to a modified AA at 41°C and 100% relative humidity for 48 h before planting (AOSA, 2009). After AA, the partially hydrated seeds were immediately transported to the field and planted in 3 by 0.76 m conventional rows with two replications. An additional row of 50 seeds per entry was planted without exposure to a modified AA as a control. Treatments were completely randomized with two replications. At harvest maturity, each entry and replicate was separately combine-threshed using an ALMACO 40 plot combine (ALMACO, IA, USA).

Seed increase

Seed of each entry harvested from both wide-spaced HC planting design and from the modified AA plots was increased in 3 by 0.76 m conventional rows, at the Iowa State University Bruner Research Farm, near Ames IA, in 2014. All entries were separately combine-harvested at maturity for the agronomic performance and seed characteristics study.

Location

In 2015, progeny from entries in wide-space HC planting design and modified AA were planted at the Iowa State University Bruner Research Farm, near Ames, IA (42°0’ 39” N, 93°43’ 50” W) on a Clarion loam (Canisteo silty clay loam) soil, and in Glidden, IA (42° 4’ 1” N, 94° 43’ 37” W) on Nicollet (Clarion silty clay loam) soil. Three hundred and twenty seeds of each entry were planted in both locations in two conventional rows of 5.3 by 0.76 m.
Treatments were arranged in a completely randomized block design blocked by location (Ames and Glidden) for testing progeny from entries grown in wide-spaced HC planting design. Entries were considered as a treatment factor. For the modified AA experiments, a split-plot design blocked by location was used with modified AA treatment as the main plot factor and entries as sub-plot factor.

**Data collection**

Data collected included plant stand, days to maturity, plant height, seed yield, 100-seed weight, standard seed germination, seed vigor, and seed oil and protein contents.

**Plant stand:** All healthy, emerged seedlings were counted at three weeks after emergence.

**Days to maturity:** Harvest maturity was determined when at least 95% of the pods attained a mature brown color (Fehr and Caviness, 1971) and data were expressed as number of days from September 1.

**Plant height:** Plant height (cm), was measured from three, randomly selected plants within each plot. Each plant was measured from the soil surface to the terminal node of the plant at harvest maturity (Fehr et al., 1977). The three plant heights were averaged to obtain plant height per entry.

**Seed yield:** Seed yield per plot was determined at harvest with an ALMACO 40 plot combine (ALMACO, IA, USA). Seed yield was recorded in kg ha\(^{-1}\) after standardizing to 13 % moisture content.

**Seed weight:** 100-seed weight (g) was calculated by averaging the weight of two sub-samples of 100-seeds per entry.
**Standard seed germination:** Seed germination was conducted according to the Association of Official Seed Analysts (AOSA) Rules for Seed Testing (2013). One hundred seeds per entry were planted on top of crepe cellulose paper (Kimberly Clark, Neenah, WI) moistened with 840 ml of water on a fiberglass tray (45 cm x 66 cm x 2.54 cm). The tray with seed was incubated at 25 °C with 8 h of light and 16 h of darkness day⁻¹. Seedling evaluation was done 7 d after planting. Seedlings were categorized into normal, abnormal, hard and dead seeds (AOSA, 2013). Values were expressed in percentages.

**Seed vigor test:** Seed vigor was determined using the accelerated aging (AA) test according to AOSA Seed Vigor Testing Handbook (AOSA, 2009) procedures. One hundred seeds per entry were placed in a single layer on a wire mesh inside a 10 x 10 x 4 cm plastic AA box containing 40 ml of distilled water. The boxes were incubated in an AA chamber (VWR Scientific Inc.) at 41°C and 100 % relative humidity for 72 h. After AA, the seeds were planted on moist crepe cellulose paper on a fiberglass tray. Four entries were planted per tray. The samples were covered with moist sand, and incubated at 25 °C with 8 h of light and 16 h of darkness day⁻¹. The percentage of normal seedlings in the AA test was evaluated 7 d after planting (AOSA, 2013).

**Seed composition:** Percentages of seed oil and protein were measured and calculated using near-infrared transmittance (NIT) using an Infratec 1229 Grain Analyzer (Foss Tecator AB, Hoganas, Sweden).

**Data analysis**

Analysis of variance was performed using the SAS PROC Mixed procedure (SAS Institute, Inc., 2012) to analyze treatment effects on agronomic performance and seed
characteristics. Data from progenies grown from seed produced in a wide-spaced HC planting design and those collected from plants whose seeds were treated with a modified AA were analyzed separately. For progeny analysis of entries in wide-spaced HC planting design, entries were considered fixed effects, while location was considered a random effect. For progeny analysis of entries in modified AA, the treatment (AA or no AA) and entries were considered fixed effects, while location was considered a random effect. Treatment means were compared using single-degree-of-freedom linear contrasts for comparison among entries within a genotype and for comparisons among genotypes. Mean differences were considered significant at $P < 0.05$.

**Results**

**Wide-spaced HC design**

Progenies from genotypes of ‘Jack’ original cultivar, 'Jack’ variant$^b$ and 'Jack’ variant$^c$ showed significant differences for all traits except plant stand, maturity and seed yield (Table 1). ‘Jack’ original cultivar was 8 ($P = 0.001$) and 20 cm ($P < 0.0001$) taller than 'Jack’ variant$^b$ and 'Jack’ variant$^c$, respectively. 'Jack’ variant$^b$ was 11 cm taller than 'Jack’ variant$^c$ ($P < 0.001$). Seed weight was approximately 1 g heavier in 'Jack’ variant$^b$ and 'Jack’ variant$^c$ than in ‘Jack’ original cultivar ($P < 0.01$), but ’Jack’ variant$^c$ weighed 0.6 g less than ’Jack’ variant$^b$ ($P = 0.01$). Standard seed germination was 3 ($P = 0.001$) and 2 % ($P < 0.05$) lower in 'Jack’ variant$^b$ and 'Jack’ variant$^c$, respectively, than in ‘Jack’ original cultivar. Seed vigor was 4 and 5 % higher in ‘Jack’ original cultivar than 'Jack’ variant$^b$ and 'Jack’ variant$^c$, respectively ($P = 0.001$). Oil content was 5.1 % lower
in ‘Jack’ original cultivar than ‘Jack’ variant\textsuperscript{b} \((P < 0.05)\). Protein content was 6.5 and 7.1% higher in ‘Jack’ original cultivar than in ‘Jack’ variant\textsuperscript{b} and ‘Jack’ variant\textsuperscript{c} \((P = 0.001)\). Progeny from entries within a genotype showed statistically significant differences for all agronomic performance and seed characteristics except plant stand and seed yield in ‘Jack’ variant\textsuperscript{b} and ‘Jack’ variant\textsuperscript{c}. Entries in ‘Jack’ original cultivar were significantly different only for plant height and protein content (Table 2).

In ‘Jack’ original cultivar, progeny from entry A58-cc was 8 cm taller than A163-cc \((P < 0.05)\), but was not different from A182-cc. In ‘Jack’ variant\textsuperscript{c}, entry A31\textsuperscript{v}-cc was 15 \((P = 0.001)\) and 17 cm \((P < 0.001)\) taller than entries A9\textsuperscript{v}-cc and A40\textsuperscript{v}-cc, respectively. No significant differences in plant height were observed among entries of ‘Jack’ variant\textsuperscript{b}.

Progeny from entries in ‘Jack’ original cultivar matured at the same time, but in ‘Jack’ variant\textsuperscript{b}, progeny from entry A13\textsuperscript{v}-bb matured 4 d later than A8\textsuperscript{v}-bb \((P < 0.01)\) and A41\textsuperscript{v}-bb \((P < 0.01)\). In ‘Jack’ variant\textsuperscript{c}, A9\textsuperscript{v}-cc matured approximately 7 and 14 d earlier than A31\textsuperscript{v}-cc and A40\textsuperscript{v}-cc, respectively, \((P < 0.0001)\). It was visually observed that the latest maturing entry in both genotypes had thicker stems and matured later than other entries (data not shown).

In ‘Jack’ variant\textsuperscript{b}, seed weight of progeny from entry A13\textsuperscript{v}-bb was 1 g heavier than both, A8\textsuperscript{v}-bb and A41\textsuperscript{v}-bb \((P < 0.05)\). In ‘Jack’ variant\textsuperscript{c}, entry A40\textsuperscript{v}-cc seed weight was 2 g heavier than both, A9\textsuperscript{v}-cc and A31\textsuperscript{v}-cc \((P < 0.001)\).

Significant seed germination differences were observed for both, ‘Jack’ variant\textsuperscript{b} and ‘Jack’ variant\textsuperscript{c}. In ‘Jack’ variant\textsuperscript{b}, progeny from entry A13\textsuperscript{v}-bb had 8 \((P < 0.001)\) and 3% \((P < 0.05)\) greater germination than A8\textsuperscript{v}-bb and A41\textsuperscript{v}-bb, respectively; while A41\textsuperscript{v}-bb
had 5% higher germination than A8\textsuperscript{v}-bb (P = 0.01). In 'Jack' variant\textsuperscript{c}, progeny from entry A9\textsuperscript{v}-cc had 6% lower germination than A31\textsuperscript{v}-cc and A40\textsuperscript{v}-cc (P = 0.01).

Significant seed vigor differences were observed in progeny from 'Jack' variant\textsuperscript{b} and 'Jack' variant\textsuperscript{c}. In 'Jack' variant\textsuperscript{b}, progeny from entries A13\textsuperscript{v}-bb and A41\textsuperscript{v}-bb had 8 (P < 0.001) and 4% (P < 0.05) greater seed vigor, respectively, than entry A8\textsuperscript{v}-bb. Entry A13\textsuperscript{v}-bb had 5% greater seed vigor than A41\textsuperscript{v}-bb (P = 0.01). In 'Jack' variant\textsuperscript{c}, entries A31\textsuperscript{v}-cc and A40\textsuperscript{v}-cc had 8 (P = 0.001) and 9% (P < 0.001) greater seed vigor, respectively, than A9\textsuperscript{v}-cc. In 'Jack' variant\textsuperscript{c}, entry A9\textsuperscript{v}-cc, which had the lowest seed vigor, also matured at least a week earlier than other entries (Table 2).

Oil content was significantly different in progeny from entries in 'Jack' variant\textsuperscript{b} but not among 'Jack' original cultivar and 'Jack' variant\textsuperscript{c}. In 'Jack' variant\textsuperscript{b}, progeny from entry A41\textsuperscript{v}-bb had 10.6% more oil than A8\textsuperscript{v}-bb and A13\textsuperscript{v}-bb (P = 0.01).

No significant differences were found for protein content among progeny from entries in 'Jack' variant\textsuperscript{b}, but among 'Jack' original cultivar and 'Jack' variant\textsuperscript{c}. In 'Jack' original cultivar, progenies from entries A182-cc and A163-cc had 1.8 and 1.3% more protein than A58-cc (P < 0.05), respectively. In 'Jack' variant\textsuperscript{c}, entries A31\textsuperscript{v}-cc and A40\textsuperscript{v}-cc had 4.0 and 5.2% more protein, respectively, than A9\textsuperscript{v}-cc (P = 0.001).

**Modified AA**

Progeny from genotypes of 'Jack' original cultivar, 'Jack' variant\textsuperscript{b} and 'Jack' variant\textsuperscript{c} showed significant differences for most traits except plant stand and standard seed germination (Table 3). Progeny from 'Jack' original cultivar was 4 (P = 0.01) and 12 cm (P < 0.0001) taller than 'Jack' variant\textsuperscript{b} and 'Jack' variant\textsuperscript{c}, respectively; while 'Jack' variant\textsuperscript{b} was 8 cm taller than 'Jack' variant\textsuperscript{c} (P < 0.0001). 'Jack' original cultivar
and 'Jack’ variant\textsuperscript{b} matured 1 ($P < 0.05$) and 2 d ($P < 0.01$) later, respectively, than 'Jack’ variant\textsuperscript{c}. Yield was 5 ($P < 0.05$) and 7\% ($P = 0.01$) lower in ‘Jack’ original cultivar than 'Jack’ variant\textsuperscript{c} and 'Jack’ variant\textsuperscript{b}, respectively. 'Jack’ variant\textsuperscript{b} and 'Jack’ variant\textsuperscript{c} seeds were 1.4 ($P < 0.0001$) and 1.2 g ($P = 0.0001$) heavier, respectively, than 'Jack’ original cultivar. Seed vigor was 5 ($P < 0.01$) and 4 \% ($P < 0.05$) greater in ‘Jack’ original cultivar than in 'Jack’ variant\textsuperscript{b} and 'Jack’ variant\textsuperscript{c}, respectively. 'Jack’ variant\textsuperscript{b} and 'Jack’ variant\textsuperscript{c} had 3.3 and 3.6 \% more oil contents than ‘Jack’ original cultivar ($P < 0.0001$). Protein content was 5.6 ($P < 0.0001$) and 6.0 \% ($P < 0.0001$) higher in ‘Jack’ original cultivar than 'Jack’ variant\textsuperscript{c} and 'Jack’ variant\textsuperscript{b}, respectively.

Significant differences were found among progeny from entries within a genotype for most agronomic performance and seed traits (Table 4). Plant height was not significantly different in progeny from entries within ‘Jack’ original cultivar, but was significant in progeny from entries within 'Jack’ variant\textsuperscript{b} and 'Jack’ variant\textsuperscript{c}. In 'Jack’ variant\textsuperscript{b}, entries A13\textsuperscript{v}-bb and A41\textsuperscript{v}-bb were 10 ($P = 0.001$) and 6 cm ($P < 0.05$) taller, respectively, than A8\textsuperscript{v}-bb. In 'Jack’ variant\textsuperscript{c}, entry A31\textsuperscript{v}-cc was 12 ($P < 0.0001$) and 9 cm ($P < 0.01$) taller than entries A9\textsuperscript{v}-cc and A40\textsuperscript{v}-cc, respectively.

Maturity in progeny from entries in all genotypes was significant. In ‘Jack’ original cultivar, progeny from entry A58-cc matured 3 ($P < 0.01$) and 2 d ($P < 0.05$) later than A163-cc and A182-cc, respectively. In 'Jack’ variant\textsuperscript{b}, entry A13\textsuperscript{v}-bb matured 4 ($P < 0.001$) and 3 d ($P = 0.001$) later than A8\textsuperscript{v}-bb and A41\textsuperscript{v}-bb, respectively. In 'Jack’ variant\textsuperscript{c}, entry A9\textsuperscript{v}-cc matured 5 ($P = 0.001$) and 9 d ($P = 0.0001$) earlier than entries A31\textsuperscript{v}-cc and A40\textsuperscript{v}-cc, respectively. Entry A31\textsuperscript{v}-cc matured 4 d earlier than A40\textsuperscript{v}-cc ($P < 0.001$).
There were no significant differences in yield for progeny from entries of ‘Jack’ original cultivar and ‘Jack’ variant, but there were differences among entries of ‘Jack’ variant. Entry A13\(^{v}\)-bb yielded 15 \((P = 0.001)\) and 12\% \((P < 0.05)\) greater than A8\(^{v}\)-bb and A41\(^{v}\)-bb, respectively. Progeny from Entry A13\(^{v}\)-bb also matured latest.

Significant 100-seed weight differences were found in progeny from entries within ‘Jack’ variant and ‘Jack’ variant entries, but not for ‘Jack’ original cultivar. In ‘Jack’ variant, entry A13\(^{v}\)-bb weighed approximately 1.4 \((P < 0.01)\) and 1.5 g \((P < 0.01)\) heavier than A8\(^{v}\)-bb and A41\(^{v}\)-bb, respectively. In ‘Jack’ variant, A40\(^{v}\)-cc weighed 1.4 \((P < 0.01)\) and 1.6 g \((P < 0.01)\) heavier than A9\(^{v}\)-cc and A31\(^{v}\)-cc, respectively.

Standard seed germination in progeny from entries within ‘Jack’ original cultivar was similar, but significant differences were observed among entries in ‘Jack’ variant and ‘Jack’ variant. In ‘Jack’ variant, entry A8\(^{v}\)-bb had 6.6 \((P < 0.05)\) and 9.6\% \((P < 0.01)\) lower seed germination than A13\(^{v}\)-bb and A41\(^{v}\)-bb, respectively. In ‘Jack’ variant, entry A9\(^{v}\)-cc had 4.4 and 6.5\% lower seed germination than A31\(^{v}\)-cc and A40\(^{v}\)-cc, respectively, \((P < 0.05)\).

Seed vigor was significantly different in progeny from ‘Jack’ variant and ‘Jack’ variant entries, but not ‘Jack’ original cultivar. In ‘Jack’ variant, progeny from entry A8\(^{v}\)-bb had 10.6 \((P = 0.001)\) and 12.6\% \((P = 0.0001)\) lower seed vigor than A13\(^{v}\)-bb and A41\(^{v}\)-bb, respectively. ‘Jack’ variant, entry A9\(^{v}\)-cc had 13.6 \((P < 0.0001)\) and 12.6\% \((P = 0.0001)\) lower seed vigor than A31\(^{v}\)-cc and A40\(^{v}\)-cc, respectively. In ‘Jack’ variant, the entry with the lowest seed vigor also matured approximately a week earlier than other entries (Table 4).
Significant differences were found in oil content for progeny from entries of 'Jack' variant\textsuperscript{b} and 'Jack' variant\textsuperscript{c} but not for 'Jack' original cultivar. In 'Jack' variant\textsuperscript{b}, progeny from entry A41\textsuperscript{y}-bb had 2.7 ($P = 0.001$) and 3.5 % ($P < 0.001$) higher oil content than A8\textsuperscript{y}-bb and A13\textsuperscript{y}-bb, respectively. 'Jack' variant\textsuperscript{c} progeny from entry A40\textsuperscript{y}-cc had 2.1 ($P < 0.05$) and 3.6 % ($P < 0.001$) lower oil content than A31\textsuperscript{y}-cc and A9\textsuperscript{y}-cc, respectively. Entry A31\textsuperscript{y}-cc had 1.5 % lower oil content than A31\textsuperscript{y}-cc ($P < 0.05$)

Protein content was significantly different for progeny from entries of 'Jack' original cultivar and 'Jack' variant\textsuperscript{c} entries, but not for 'Jack' variant\textsuperscript{b}. In 'Jack' original cultivar, progeny from entry A58-cc had 2.4 ($P < 0.001$) and 3.1% ($P < 0.0001$) lower protein content than A163-cc and A182-cc, respectively. In 'Jack' variant\textsuperscript{c}, entry A9\textsuperscript{y}-cc progeny had 1.8 ($P = 0.01$) and 3.4 % ($P < 0.0001$) less protein than A31\textsuperscript{y}-cc and A40\textsuperscript{y}-cc, respectively. Progeny from entry A31\textsuperscript{y}-cc had 1.7 % ($P = 0.01$) less protein than A40\textsuperscript{y}-cc.

There were no significant interactions among progeny from all entries and modified AA treatment for agronomic performance and seed characteristics. Therefore, data were analyzed across entries for all traits. Results showed no significant differences between modified AA and the control for all traits except plant stand, plant height and seed yield. Progeny from entries in the control emerged better (9 more emerged plants) ($P < 0.05$) than progeny from entries exposed to modified AA treatment. The control plants were approximately 3 cm ($P < 0.05$) taller and yielded approximately 4% ($P < 0.05$) lower than progeny from modified AA-treated plants (Table 5).
Discussion

Genotypes and entries had similar responses to the wide-spaced HC planting design (Tables 1 and 2) and the modified AA (Tables 3 and 4). Genotypes and entries that had low agronomic performance and seed characteristics in wide-spaced HC planting design, such as plant height, maturity, 100-seed weight, seed vigor and oil content, also had similar responses to a modified AA. However, the overall performance of genotypes and entries in wide-spaced HC planting design was better than those in modified AA. For instance, plant height and yield were over 100 cm and 3000 kg ha\(^{-1}\), respectively, in progeny grown from plants in wide-spaced HC design, while plants were shorter (<100 cm) and yielded less (<3000 kg ha\(^{-1}\)) than progeny from entries treated with a modified AA. In our study, modified AA negatively affected agronomic performance and seed characteristics of the genotypes and entries. This negative effect carried over to the progeny. The wide-spaced HC planting design was used in breeding and selection because it allowed full expression of traits by eliminating interplant competition (Fasoulas and Fasoula, 1995; 1997). Our research confirmed that progeny from a wide-spaced HC design had better performance overall.

‘Jack’ original cultivar progeny were taller than ’Jack’ variant\(^b\) and ’Jack’ variant\(^c\), which were shorter and had thicker stems especially in late-maturing entries. We visually observed that ‘Jack’ original cultivar lodged significantly more than ’Jack’ variant\(^b\) and ’Jack’ variant\(^c\) genotypes (data not shown), which could be the result of taller plants (Wilcox and Sediyama, 1981; Curtis et al., 2000). Resistance to lodging is an important trait for reducing yield loss and for facilitating mechanical harvesting (Weber
and Fehr, 1966; Wilcox and Sediyama, 1981). Therefore, these entries could be a useful source of lodging tolerance once fully characterized.

Maturity was significantly different in progeny from genotypes exposed to a modified AA, where 'Jack' variant c matured earlier than 'Jack' original cultivar and 'Jack' variant b, although no significant difference were observed in progeny from plants wide-spaced HC planting design (Tables 1 and 3). Maturity may be an important trait in different production ecosystems. For example, in areas with a dry season, farmers may prefer early maturing soybean cultivars to escape the drier months. In areas prone to early fall frosts, early maturing lines may escape frost damage allowing the production of high quality seed. Our results showed that these early maturing genotypes also have higher yield, an essential trait in soybean cultivar improvement.

Seed yield was significantly higher in 'Jack' variant b and 'Jack' variant c than in 'Jack' original cultivar, although no significant differences were observed in progeny from wide-spaced HC design. Seeds from these genotypes were heavier (>100-seed weight) than 'Jack' original cultivar, which might have contributed to higher yields as evidenced by a positive relationship between the two traits (Figs. 2A and 2B). Our findings are in agreement with previous work that reported a positive relationship between seed yield and seed weight in soybean (Meckel et al., 1984; Specht et.al., 1986; Fasoula and Boerma, 2007). Also, the progeny from these variants planted in a wide-spaced HC yielded progressively better as 100-seed weight increased, until reaching a plateau before yield decreased with increasing 100-seed weight. In Contrast, the 100-seed weights of progeny from 'Jack' variants exposed to a modified AA were lighter but yield increased proportional to 100-seed weight increase. This 100-seed weight
difference between variants in a wide-spaced HC versus exposed to modified AA could be the result of lower seed vigor (Delouche and Baskin, 1973). However, our research is first to report lighter 100-seed weight in progeny from artificially aged seed. Further research should evaluate if these differences in 100-seed weight and yield remain stable in subsequent generations.

Standard seed germination and seed vigor were significantly lower in progeny from 'Jack' variant\textsuperscript{b} and 'Jack' variant\textsuperscript{c} than in 'Jack' original cultivar, although the differences were not significant in progeny from plants exposed to a modified AA. The progeny of variants had higher oil and lower protein content than 'Jack' original cultivar. Other authors have reported an inverse relationship between oil and protein content in seed (Filho et al., 2001). Seed oils, especially those rich in unsaturated fatty acids, are susceptible to oxidative deterioration leading to loss of viability and vigor. The reactive oxidation of fatty acids is associated with the normal aging process in seeds (Parrish and Leopold, 1978; McDonald 1999; Ghassemi-Golezani et al., 2010), and has been reported in seed after AA (Kumar et al., 2015). Stored seeds with higher oil content rapidly deteriorated and lost seed vigor sooner than high protein seeds (Mbofung et al., 2013). Since seeds from our experiments were freshly harvested, the lower seed germination and vigor seemed associated to metabolic changes in these variants.

The low standard germination and seed vigor could be the result of mechanical damage associated with larger seed size and low moisture content. Progeny from genotypes 'Jack' variant\textsuperscript{b} and 'Jack' variant\textsuperscript{c} had significantly larger seed sizes than 'Jack' original cultivar (Tables 2 and 4). The progeny of these variants also matured earlier. Consequently, seeds could have been drier at harvest. Other studies have
reported higher levels of mechanical damage in dry beans (Wijandi and Copeland, 1974) and soybean (Misra et al., 1985; Rahman et al., 2004).

Although seed quality in our study was lower in ‘Jack’ variant$^b$ and ‘Jack’ variant$^c$ than ‘Jack’ original cultivar, the germination percentages of these progenies were still within the recommended level for adequate seedling emergence and stand establishment of $\geq 80\%$ (Egli and Tekrony, 1995). The minimum seed vigor in our study was 82\% (Table 3).

We found significant variability in progeny from entries within ‘Jack’ variant$^b$ and ‘Jack’ variant$^c$ for both, wide-spaced HC design and the modified AA experiment. Mean plant height, maturity, 100-seed weight, seed vigor, seed germination, oil and protein contents for entries within ‘Jack’ variant$^b$ and ‘Jack’ variant$^c$ were significantly different compared with entries from ‘Jack’ original cultivar. Only plant height and protein content in progeny from entries in a wide-spaced HC design, and maturity and protein in progeny from entries treated with a modified AA were significantly different in ‘Jack’ original cultivar (Tables 2 and 4).

Genetic variation has been reported for agronomic performance and seed characteristics in cultivars considered nearly homozygous (Christakis and Fasoulas, 2002; Fasoula and Boerma, 2005, 2007; and Tokatlidis, 2011; Espinosa et al., 2015; Sandhu et al., 2016). This genetic variation has been attributed to pre-existing variation in parental lines (Haun et al., 2011; Yates et al., 2012), newly-induced variation (Hawbaker et al., 1993; Fasoula and Boerma, 2005, 2007; Yates et al., 2012), or epigenetic changes (Kovalchuk et al., 2003; Hauben, et al., 2009). The genetic variation also has been
attributed to genome plasticity or reorganization of the genome when plants are exposed to stimulating environments (McClintock, 1984; Rasmusson and Phillip, 1997).

Fasoula and Boerma (2005, 2007) found genetic variability for seed composition and seed weight in lines selected from three commercial soybean cultivars. Cultivars Benning, Cook and Haskell were planted in wide-spaced HC planting design, evaluated, and 19 lines unique lines were identified. When these lines were evaluated using simple sequence repeat (SSR) markers (Yates et al., 2012), much of the variation could be traced to foundation seed source and original parents. However, some of the genetic variation observed could not be traced to the original seed source. This genetic variation was attributed to mutations or de novo variation.

Hawbaker et al. (1993) induced variations in agronomic performance and seed characteristics in three soybean cultivars through tissue culture. The progeny lines from these cultivars had higher and lower seed yield, seed weights, oil contents and matured earlier or later than the control plants. The authors suggested that tissue culture induced new genetic variation. Similar tissue-culture induced genetic variation has been reported in flax (Durrant, 1962; Cullis et al., 1999) and canola (Hauben, et al., 2009).

In our study, we found less variation in agronomic performance and seed characteristics in progeny from ‘Jack’ original cultivar entries than in ‘Jack’ variant\(^b\) and ‘Jack’ variant\(^c\) entries. Therefore, it is unlikely that all the observed variation could be pre-existing in the original seed source, ‘Jack’ original cultivar. Future studies should explore a genetic characterization of the variants to determine if the observed phenotypic variation is due to genetic changes at the \(Aco4\) gene loci (Espinosa, et al., 2015), among others.
The modified AA had a negative effect on agronomic performance. AA is a stress treatment that subjects the seed to high temperature and relative humidity, affecting stability of sugars and reactive oxygen scavenging enzymes (Bernal-Lugo and Leopold, 1998; McDonald, 1999; Ghassemi-Golezani et al., 2010; Marcos-Filho, 2015). Seed aging has a negative effect on agronomic performance and has been associated with metabolic changes of increased lipid peroxidation, reduced antioxidants and activity of free radical scavenging enzymes, and damage to nucleic acid and proteins (Parrish and Leopold, 1978; Bernal-Lugo and Leopold 1998; McDonald, 1999; Rolling, 2012; Kumar, et al., 2015). Rolling (2012) found that soybean seed exposed to modified AA had reduced plant stand and plant height. However, in her study, the plants had higher yield per plot compared to the control. Similar results were reported in sesame seed (Tabatabaei, 2013), maize seed (Ghassemi-Golezani et al., 2011) winter oilseed rape (Ghassemi-Golezani et al., 2010), barley (Rajala et al., 2011) and lentils (Ghassemi-Golezani et al., 2014). Reduced plant height and higher yield per plot has been attributed to reduced competition for growth resources such as moisture, sunlight, and nutrients resulting in faster crop growth rate, greater branch and seed dry matter, and increased pod number per plant (Wells, 1993; Carpenter and Board, 1997; Saha and Sultana, 2008). However, our research indicated that progeny from these plants also experience measurable agronomic changes. These results may be explained by genetic changes or epigenetic stress-memory that could be retained in the seed of the stressed generation, and be expressed in the progeny without additional exposure to stimulating environments (Migicovsky et al., 2014). Further testing of these theories would confirm this assertion.
Conclusion

This study evaluated agronomic performance, seed characteristics and variability in progeny from entries of 'Jack' variant\(^b\), 'Jack' variant\(^c\), and 'Jack' original cultivar planted in a wide-spaced HC planting design or exposed to a modified AA. Our results showed that agronomic performance and seed characteristics of progeny from entries within 'Jack' variant\(^b\) and 'Jack' variant\(^c\) were more variable than those in Jack‘ original cultivar for almost all traits evaluated. These variants had higher yield, oil content, shorter or longer maturity, and other agronomic and seed characteristics that could be used to develop new cultivars. Further research into the stability of these traits over generations will determine their usefulness as new sources of germplasm.

Acknowledgements

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References


### Tables and Figures

Table 1. Means for plant stand, plant height, maturity, yield, 100-seed weight, standard germination, seed vigor, oil, and protein content of progenies of three soybean genotypes from wide-spaced HC planting design

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Plant Stand (Number of emerged plants)</th>
<th>Plant Height (Cm)</th>
<th>Maturity (days from Sept 1)</th>
<th>Yield (Kg ha(^{-1}))</th>
<th>Seed weight (g 100(^{-1}))</th>
<th>Standard germination (%)</th>
<th>Seed Vigor (%)</th>
<th>Oil (%)</th>
<th>Protein (%)</th>
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<tbody>
<tr>
<td>Original ‘Jack’</td>
<td>244</td>
<td>120.37 a(^1)</td>
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<td>3108.74</td>
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<td>91 a</td>
<td>86 a</td>
<td>18.61 b</td>
<td>35.86 a</td>
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<td>3369.69</td>
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<td>88 b</td>
<td>82 b</td>
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<td>‘Jack’ variant(^c)</td>
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<td>3206.49</td>
<td>13.95 b</td>
<td>89 b</td>
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<td>19.28 ab</td>
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<td>±1</td>
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<td>±1</td>
<td>±1</td>
<td>±0.36</td>
<td>±0.15</td>
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\(^{\text{a}}\)Means followed by the same letter within a column per variant type are not significantly different at \(P < 0.05\)
Table 2. Means for plant stand, plant height, maturity, yield, 100-seed weight, standard germination, seed vigor, oil, and protein content of progenies of entries of the three soybean genotypes from wide-spaced HC planting design

<table>
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<tr>
<th>Genotype</th>
<th>Entry</th>
<th>Plant Stand (Number of emerged plants)</th>
<th>Plant Height (Cm)</th>
<th>Maturity (days from Sept 1)</th>
<th>Yield Kg ha(^{-1})</th>
<th>Seed weight (g 100(^{-1}))</th>
<th>Standard germination (%)</th>
<th>Seed Vigor (%)</th>
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<td>SE</td>
<td>±7</td>
<td>±2.99</td>
<td>±1</td>
<td>±163.03</td>
<td>±0.32</td>
<td>±1</td>
<td>±1</td>
<td>±0.63</td>
<td>±0.26</td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\)Means followed by the same letter within a column per genotype are not significantly different at \(P < 0.05\)
Table 3. Means for plant stand, plant height, maturity, yield, 100-seed weight, standard germination, seed vigor, oil, and protein content of progenies of three soybean genotypes from modified AA

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Plant stand (number of emerged plants)</th>
<th>Plant Height (Cm)</th>
<th>Maturity (days from Sept 1)</th>
<th>Yield Kg ha(^{-1})</th>
<th>Seed weight (g 100(^{-1}))</th>
<th>Standard germination (%)</th>
<th>Seed Vigor (%)</th>
<th>Oil (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Jack’ original</td>
<td>255</td>
<td>98.37 a(^{1})</td>
<td>31 a</td>
<td>2408.11 b</td>
<td>12.21 b</td>
<td>92</td>
<td>87 a</td>
<td>18.73 b</td>
<td>35.58 a</td>
</tr>
<tr>
<td>‘Jack’ variant(^{b})</td>
<td>255</td>
<td>94.58 b</td>
<td>31 a</td>
<td>2576.88 a</td>
<td>13.63 a</td>
<td>90</td>
<td>83 b</td>
<td>19.40 a</td>
<td>33.57 b</td>
</tr>
<tr>
<td>‘Jack’ variant(^{c})</td>
<td>256</td>
<td>86.11 c</td>
<td>29 b</td>
<td>2532.38 a</td>
<td>13.43 a</td>
<td>89</td>
<td>84 b</td>
<td>19.35 a</td>
<td>33.70 b</td>
</tr>
<tr>
<td>SE</td>
<td>±4</td>
<td>±1.40</td>
<td>±1</td>
<td>±52.89</td>
<td>±0.25</td>
<td>±1</td>
<td>±1</td>
<td>±0.10</td>
<td>±0.12</td>
</tr>
</tbody>
</table>

\(^{1}\)Means followed by the same letter within a column are not significantly different at \(P < 0.05\)
Table 4. Means for plant stand, plant height, maturity, yield, 100-seed weight, standard germination, seed vigor, oil, and protein content of progenies of entries of three soybean genotypes from modified AA

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Entry</th>
<th>Plant Stand (Number of emerged plants)</th>
<th>Plant Height (Cm)</th>
<th>Maturity (days from Sept 1)</th>
<th>Yield Kg ha(^{-1})</th>
<th>Seed weight (g 100(^{-1}))</th>
<th>Standard germination (%)</th>
<th>Seed Vigor (%)</th>
<th>Oil (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Jack' original</td>
<td>A58-cc</td>
<td>253</td>
<td>100.52</td>
<td>32 a</td>
<td>2418.19</td>
<td>12.23</td>
<td>94</td>
<td>87</td>
<td>18.83</td>
<td>34.91 b</td>
</tr>
<tr>
<td></td>
<td>A163-cc</td>
<td>257</td>
<td>98.75</td>
<td>29 b</td>
<td>2451.46</td>
<td>11.94</td>
<td>90</td>
<td>85</td>
<td>18.74</td>
<td>35.78 a</td>
</tr>
<tr>
<td></td>
<td>A182-cc</td>
<td>254</td>
<td>95.83</td>
<td>30 b</td>
<td>2354.67</td>
<td>12.44</td>
<td>91</td>
<td>89</td>
<td>18.64</td>
<td>36.04 a</td>
</tr>
<tr>
<td>'Jack' variant(^{b})</td>
<td>A8(^{v})-bb</td>
<td>255</td>
<td>88.96 b(^{i})</td>
<td>29 b</td>
<td>2428.45 b</td>
<td>13.17(^{b})</td>
<td>85 b</td>
<td>76 b</td>
<td>19.28 b</td>
<td>33.36</td>
</tr>
<tr>
<td></td>
<td>A13(^{v})-bb</td>
<td>251</td>
<td>99.38 a</td>
<td>33 a</td>
<td>2799.74 a</td>
<td>14.61(^{a})</td>
<td>94 a</td>
<td>85 a</td>
<td>19.11 b</td>
<td>33.68</td>
</tr>
<tr>
<td></td>
<td>A41(^{v})-bb</td>
<td>261</td>
<td>95.42 a</td>
<td>30 b</td>
<td>2502.45 b</td>
<td>13.11(^{b})</td>
<td>91 a</td>
<td>87 a</td>
<td>19.81 a</td>
<td>33.67</td>
</tr>
<tr>
<td>'Jack' variant(^{c})</td>
<td>A9(^{v})-cc</td>
<td>262</td>
<td>80.52 b</td>
<td>24 c</td>
<td>2482.70</td>
<td>12.86(^{b})</td>
<td>86 b</td>
<td>76 c</td>
<td>19.69 a</td>
<td>33.10 c</td>
</tr>
<tr>
<td></td>
<td>A31(^{v})-cc</td>
<td>256</td>
<td>93.33 a</td>
<td>30 b</td>
<td>2470.54</td>
<td>12.94(^{b})</td>
<td>90 a</td>
<td>88 a</td>
<td>19.39 b</td>
<td>33.71 b</td>
</tr>
<tr>
<td></td>
<td>A40(^{v})-cc</td>
<td>251</td>
<td>84.48 b</td>
<td>33 a</td>
<td>2643.90</td>
<td>14.50(^{a})</td>
<td>92 a</td>
<td>87 a</td>
<td>18.99 c</td>
<td>34.28 a</td>
</tr>
<tr>
<td>SE</td>
<td>±7</td>
<td>+2.42</td>
<td>±1</td>
<td>+91.61</td>
<td>±0.43</td>
<td>±2</td>
<td>±2</td>
<td>±0.17</td>
<td>±0.20</td>
<td></td>
</tr>
</tbody>
</table>

\(^{i}\)Means followed by the same letter within a column per genotype are not significantly different at \(P < 0.05\)
Table 5. Means for plant stand, plant height, maturity, yield, 100-seed weight, standard germination, seed vigor, oil, and protein content of aging or no aging treatment across genotypes

<table>
<thead>
<tr>
<th>Trait</th>
<th>Aging</th>
<th>No aging</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stand (Number of emerged plants)</td>
<td>251.00 b†</td>
<td>260.00 a</td>
<td>±6.00</td>
</tr>
<tr>
<td>Yield (Kgha-1)</td>
<td>2552.02 a</td>
<td>2459.56 b</td>
<td>±947.14</td>
</tr>
<tr>
<td>Height (Cm)</td>
<td>91.76 b</td>
<td>94.28 a</td>
<td>±18.47</td>
</tr>
<tr>
<td>Maturity (Days from Sept 1)</td>
<td>30.00</td>
<td>30.00</td>
<td>±1.00</td>
</tr>
<tr>
<td>Seed weight (g 100-1)</td>
<td>13.26</td>
<td>12.91</td>
<td>±1.14</td>
</tr>
<tr>
<td>Seed Vigor (%)</td>
<td>85.00</td>
<td>84.00</td>
<td>±1.00</td>
</tr>
<tr>
<td>Standard germination (%)</td>
<td>90.00</td>
<td>90.00</td>
<td>±1.00</td>
</tr>
<tr>
<td>Oil (%)</td>
<td>19.18</td>
<td>19.14</td>
<td>±0.37</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>34.32</td>
<td>34.24</td>
<td>±0.50</td>
</tr>
</tbody>
</table>

†Means followed by different letter within a row are significantly different at \( P < 0.05 \)
Honeycomb (HC)
‘Jack’ (Aco4-cc) and five other cultivars (500 plants (entries)).

Accelerated aging (AA)
64 entries planted in rows in 2 reps. Collected 3 (3-seeded) pods per entry/rep.

Aconitase-4 (Aco4) isozyme test
3-seeded pods (1152 seeds tested); 1 unusual Aco4-bc; Entry 127 and 4 other variants

Stability test
Selfed Aco4-bc plants. Generated Aco4-bb and Aco4-cc.

Seed increase
Aco4-bb and Aco4-cc variants 50 entries

Allelism test
Jack Aco4-cc x Minsoy Aco4-cc.

HC
3 entries/variant and Original Jack (Aco4-cc) at 25 seeds/entry 225 single 3-seeded pods, one pod per plant

Aconitase test
5 seeds of each entry To confirm aco4 alleles

AA
3 entries/variant and Original Jack (Aco4-cc), 2 reps. 50 seeds/entry/rep. 108 single 3-seeded pod from 3 plants/entry/rep.

Figure. 1. Seed source for cultivar ‘Jack’ variants for agronomic performance and seed characteristics experiment conducted from 2013 to 2015. A solid bold line indicates the initiation of this experiment (2013)
Figure. 2. Relationship between mean 100-seed weight and seed yield in progenies of ‘Jack’ variants from; (A) wide-spaced HC design, and (B) modified AA before planting in rows. Progeny plants in both treatments were evaluated in conventional row in 2015.
CHAPTER 4. ACONITASE-4 ALLELE STABILITY IN SOYBEAN ‘JACK’ VARIANTS EXPOSED TO ADDITIONAL ENVIRONMENTAL STIMULI

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Abstract

Environmental stimuli generated new soybean variants in nearly homozygous cultivars. These new variants could be useful for new cultivar development. For these variants to be useful in plant breeding, however, genotypic and phenotypic stability over generations and after additional environmental stimuli is critical. Climate change and other environmental challenges, such as pollutant toxicities and water deficits, could influence genetic makeup of unstable variants. Thus, it is important to understand the genetic mechanisms behind the generation of these variants. The Aconitase-4 (Aco4) isozymes variants had an allele switching event in a single seed from a three seeded pod. The objective of this study was to evaluate the stability of these Aco4 isozymes soybean variants in cultivar ‘Jack’ seeds from plants grown in a wide-spaced honeycomb (HC) planting design and plants from seeds exposed to modified accelerated aging (AA) before planting. The second objective was to determine genetic differences between these variants and ‘Jack’ original foundation seed at the Aco4 gene, Glyma.11g080600. In this study, we identified two new Aco4 isozyme double allele-switching in one seed within a
three-seeded pod in two entries. Entry A13\(^{y}\)-bb plant 183 seed number one (A13\(^{y}\)-bb-183-1) changed from Aco4-bb to Aco4-cc, and entry A9\(^{y}\)-cc plant 229 seed number three (A9\(^{y}\)-cc-229-3) changed from Aco4-cc to Aco4-bb out of 75 seeds per entry. We also identified one seed with a single allele-switching from Aco4-bb to Aco4-bc in entry A13\(^{y}\)-bb plant 67 seed number one (A13\(^{y}\)-bb-67-1), where the ‘c’ allele was unexpected. All new variants were found in seeds from plants grown in a wide-spaced HC planting design and not from plants where seeds were exposed to modified AA before planting. We identified nucleotide substitutions in the exons of Glyma.11g080600 that resulted in amino acid substitution in the proteins of Aco4-bb and Aco4-cc samples. This SNPs differences could explain differences observed in the Aco4 isozyme patterns. These results indicated that the aconitase allele switching was caused by with genetic changes. This new genetic variation in nearly homozygous soybean cultivars may explain the continuous adaptation process plants undergo to survive changes in the environment. The wide-spaced HC planting design may be a valuable tool for generating intra-cultivar genetic variation. Plant breeders could use wide-spaced HC planting design to induce novel genetic variation which could be useful for cultivar development.

**Introduction**

The genetic and phenotypic variation in nearly homozygous soybean cultivars is considered not existent (Roth et al., 1989; Fasoula and Boerma, 2005, 2007; Yates et al., 2012). However numerous studies have discovered within-cultivar genotypic and phenotypic variation in soybean and other crops, which were stably inherited across generations (Durrant, 1962; Roth et al., 1989; Brettel et al., 1991; Amberger et al., 1992a,
This variation has been attributed to the fluidity nature of the genome and could be due to genetic (Walbot and Cullis, 1985; Brettel et al., 1986; Yates et al. 2012), epigenetic (Walbot and Cullis, 1985; Boyko and Kovalchuk, 2011; Migicovsky et al., 2014), morphological, or physiological (Taiz and Zeiger 2010) changes that enable plants to adapt to stressful environmental conditions (McClintock, 1984; Ceccarelli et al., 1997; Rasmusson and Phillips, 1997; Casacuberta and Puigdomènech, 2000).

Previous studies have reported heritable genetic variation induced by tissue culture stress in soybean and other crops. Amberger et al. (1992a, 1992b) found two heritable isozyme variants in 185 soybean plants raised from tissue culture. One variant was an aconitase 2 isozyme null mutant, and the other was a chlorophyll-deficient plant, which lacked two mitochondrial malate-dehydrogenases. This variation was not present in the control plants and had never been reported before in soybean. The authors attributed this variation to deletion or activation of transposable elements that led to deletions and other DNA rearrangement triggered by tissue culture stress. Using restriction fragment length polymorphism (RFLP), Roth et al. (1989) detected new alleles in root tissues of two soybean cultivars, ‘Minsoy’ and ‘Noir,’ and their crosses subjected to tissue culture stress. Strikingly, these alleles were present in other soybean cultivars but not in these two cultivars. The authors hypothesized that the genome from nearly homozygous soybean cultivars remained fluid in order to adapt to environmental stimuli. Within-cultivar genetic and phenotypic variation for seed oil, protein, seed weight, maturity and other traits were observed in ‘Benning,’ ‘Cook’ and ‘Haskell’ soybeans cultivars grown in a wide-spaced honeycomb (HC) planting design (Fasoula and
Boerma, 2005, 2007). DNA analysis of these plants with simple sequence repeat (SSR) markers showed that most of the variation was pre-existing in original parents and foundation seed sources (Yates et al., 2012). However, some variation could not be traced back to the original parents and the foundation seed source. This variation was attributed to newly induced genetic variation (Yates et al., 2012).

Recent studies identified aconitase isozymes allelic variation in soybean homozygous cultivars exposed to environmental stimuli (Espinosa et al., 2015; Coleman et al., 2016). Aconitase (aconitate hydratase enzyme commission (EC) 4.2.1.3) catalyzes the reverse interconversion of citrate to isocitrate via a cis-aconitic acid intermediate in the Krebs cycle (Rouault and Klausner 1996; Sadka et al., 2000; Shlizerman et al., 2007). Five aconitase isozymes, namely Aco1, Aco2, Aco3, Aco4 and Aco5, have been identified in soybeans (Griffin and Palmer, 1987; Kiang and Bult 1991; Terol et al., 2010; Espinosa et al., 2015). Aco4 gene (Glyma.11g080600) was mapped to chromosome 11 in the soybean genome (Espinosa et al., 2015, Coleman et al., 2016). The gene is 8362 bp long and has 20 exons that code for 901 amino acids long protein (www.phytozome.net).

Espinosa et. al. (2015) also reported a single-allele switch from the usual Aco4-cc to Aco4-bc in one seed of a three-seeded pod collected from cultivar ‘Jack’. In addition, the authors also reported finding four seeds with a double allele switch from Aco4 cc to Aco4-bb. Seed source for the experiment was from a plant that was single-plant threshed, grown in wide-spaced HC planting design exposed to natural hail at V4-V5 and whose seed was subjected to a modified accelerated aging (AA) before planting. Self-pollination of Aco4-bc showed that the alleles segregated according to the Mendelian ratio. Stability studies with Aco4-bb and Aco4-cc variants indicated that they were stable and heritable.
For these variants to be useful in plant breeding, stability over generations and after additional environmental stimuli is critical. Climate change and other environmental challenges, such as pollutant toxicities and water deficits, should not influence genetic makeup or stability of these variants if used as production varieties. Furthermore, understanding the mechanisms behind allele switching in single seed from a three-seeded pod in soybean cultivars would facilitate using these variants in plant breeding.

The objectives of this study were to evaluate the stability of the Aco4 isozymes in soybean seed from ‘Jack’ variants exposed to either a wide-spaced HC planting design or modified AA before planting; and to determine genetic differences between variants and ‘Jack’ original foundation seed for Glyma.11g080600.

Materials and Methods

Seed source and experimental design

Seed for this study was obtained from an experiment initiated in 2008 (Espinosa et al., 2015) and was summarized in Table 1; Fig. 2. The original Espinosa et al. (2015) experiment was initiated in 2008 using seed obtained from Dr. Randy Shoemaker, USDA ARS-Iowa State University, Ames, IA (Fig. 2). In this original study, five cultivars of soybean, including ’Jack’, were grown in a wide-spaced HC planting design. During the growing season, plants were severely damaged by a hail storm, and yield in most plants was reduced. However, seed from a few surviving plants were single-plant threshed and assigned a number that denominated “entry”. In 2009, 64 of these entries from ‘Jack’ cultivars were stressed using a modified AA. The modified AA treatment consisted of exposing soybean seeds to 41°C temperature, and 100% relative humidity
for 48 h, instead of the recommended 72 h (AOSA, 2009) before field planting. Two replications per entry were planted in conventional rows. At harvest maturity three, 3-seeded pods were collected from three randomly selected plants per cultivar, per entry, and per replication. The aconitase isozyme test was conducted on every seed from all pods using starch-gel electrophoresis for a total of 1152 seeds per cultivar. Results from the test indicated that a single-allele switch had occurred from the usual $Aco4$-$cc$ to $Aco4$-$bc$, in one seed of a 3-seeded pod collected from ‘Jack’ entry 127 replicate 1, in addition to four seeds that had double allele-switching from $Aco4$-$cc$ to $Aco4$-$bb$ (Espinosa et al., 2015). The $Aco4$-$bc$ plant was self-pollinated and alleles segregated in a normal $1bb:2bc:1cc$ ratio. Stability studies with $Aco4$-$bb$ and $Aco4$-$cc$ variants, denoted in this study as 'Jack’ variant$^b$ and 'Jack’ variant$^c$, indicated that the alleles were heritable and stable (Espinosa, et al., 2015). In 2011, 50 plants per variant were single plant-threshed and each plant was assigned an entry number. In 2012, seed from the 50 entries per variant were increased for subsequent studies.

Our study was initiated in 2013 and used three genotypes from Espinosa et al. (2015). Three entries per variant (Variants $Aco4$-$bb$ and $Aco4$-$cc$) and three other entries from ‘Jack’ original cultivar with $Aco4$-$cc$ from Espinosa et al. (2015) were used in this study to evaluate stability of the $Aco4$ isozyme alleles when exposed to additional stimuli (Fig. 2). The genotypes were: ‘Jack’ original cultivar, 'Jack’ variant$^b$ and 'Jack’ variant$^c$. Genotypes were named as follows: cultivar and type (original or variant) and super-script b and c indicated allelic patterns bb or cc. Two separate experiments were conducted to evaluate the stability of these variants, a wide-spaced HC planting design and a modified AA. Each genotype had three entries according to the 2008 experiment
nomenclature (Espinosa et al., 2015). The nomenclature used to identify the entries in this study were: ‘A’ indicated aconitase gene; entry number indicated plant number in the original experiment; type was indicated by a superscript, no superscript beside the plant number indicated ‘Jack’ original type and a superscript (v) indicated a variant; and bb or cc indicated the Aco4 isozyme allelic pattern. The entries of ‘Jack’ variant included in this study were A8-v-bb, A13-v-bb and A41-v-bb; the entries of ‘Jack’ variant were A9-v-cc, A31-v-cc and A40-v-cc; and the entries of the ‘Jack’ original cultivar were A58-cc, A163-cc and A182-cc.

Wide-spaced HC planting design

In 2013, a total of 25 seeds of each of the nine entries (Table 1; Fig. 2) were wide-space planted in a HC pattern at a plant-to-plant spacing of 2.8 m, for a total of 225 plants. These plants were assigned plant numbers from 37-261. For instance, the nomenclature used to identify the entries and plants in this study were A13-v-bb-41, where ‘A’ indicated the aconitase gene; ‘13’ indicated the entry number from the original 2008 experiment; genotype was indicated beside the 2008 entry number by a superscript (v) for variant or no superscript for the ‘Jack’ original cultivar; bb or cc indicated the allelic pattern; and the new 2013 entry number was indicated by plant number, ‘41’. Four seeds were planted per hill, and thinned to one healthy plant at V2-V3.

In 2014, 50 seedlings randomly selected from those tested for Aco4 isozyme collected in 2013, including two new variants (Aco4-bb to Aco4-cc and Aco4-cc to Aco4-bb), were transplanted into peat pots containing a standard greenhouse soil mix (1 sand: 1 peat moss: 2 soil) in the USDA-ARS greenhouse at Ames, IA. At the beginning of the growing season, the seedlings were transplanted to the field in a wide-spaced HC planting
design (Fig. 2). These plants maintained the same plant numbers assigned in 2013. In 2015, seed from 2013 was used to plant a second wide-spaced HC planting design with 12 seeds per entry, for a total of 108 plants. New plant numbers were assigned to each plant from 1-108, following the same nomenclature used in 2013.

**Modified AA**

In 2013, 50 seeds from each of the nine entries were exposed to a modified AA at 41°C and 100 % relative humidity for 48 h before planting (AOSA, 2009). After AA, the partially hydrated seeds were immediately transported to the field and planted in 3 by 0.76 m conventional rows with two replications. An additional row of 50 seeds per entry was planted without exposure to a modified AA as a control. In 2014, the modified AA experiment was repeated under the same conditions as in 2013.

**Seed sample collection from wide-spaced HC planting design**

In 2013 and 2015, a single three-seeded pod was collected from each plant grown in the wide-spaced HC planting design for Aco4 isozyme test (Table 1; Fig. 2). According to the number of plants per entry each year, 25 and 12 three-seeded pods were collected per entry in 2013 and 2015 for a total of 225 and 108 pods, respectively. In 2014, three 3-seeded pods per new Aco4-isozyme variant and a single, three-seeded pod from each of the remaining plants were collected for a total of 54 pods. Pods were stored at 10 °C until Aco4 isozyme tests were conducted.
Seed sample collection from modified AA

In 2013 and 2014, one 3-seeded pod each from three randomly selected plants per entry per treatment (modified AA or no modified AA) and per replication were collected. A total of 108 three-seeded pods were collected in each year. Pods were stored at 10 °C until Aco4 isozyme test was conducted.

Aco4 Isozyme test

The Aco4 isozyme test was performed on each seed using starch-gel electrophoresis (Cardy and Beversdorf, 1984). A total of 675, 162 and 324 seeds were tested in 2013, 2014 and 2015, respectively, for the wide-spaced HC planting design experiment. A total of 324 seeds were tested each year (2013 and 2014) from the modified AA experiment. Seeds inside the pod were numbered 1 to 3, where seed number 1 was the closest to the main stem.

Seed germination and cotyledon sampling

Seed were germinated on a labeled, moistened germination paper in a dark growth chamber maintained at 28 to 30 °C for 3 d (Griffin and Palmer, 1987). Each entry was separately tested to avoid artifacts. Three pieces from the tip of one cotyledon of each seedling were sampled using a 200 µL glass-bore pipette tube, and inserted into a labelled 1.5 mL polypropylene microcentrifuge tube containing 125 µL of cold homogenization buffer [0.1M tris-HCl (pH 7.2), 4% (wt/vol) PVP-40 (polyvinylpyrrolidone, molecular weight 40,000), 400 mM sucrose, and 1 mM dithiothreitol added just before use]. Seedlings were rolled back on germination paper towels and placed in a lighted growth chamber to photosynthesize and grow for 2-3 d before transplanting into peat pots containing a standard greenhouse soil mix (1 sand: 1 peat moss: 2 soil).
Sample homogenization and electrophoresis

Samples were homogenized using a pointed acrylic pestle, held by an electric motorized stir (TRI-R STIR-R, Model S63C, Chicago, IL) that fit inside the 1.5 mL microcentrifuge tube. Samples were homogenized for 45 sec or until a uniform pulp was obtained. The pestle was rinsed in pure water between samples. After homogenization, sample tubes were centrifuged at 4 °C at 14000 rpm for 6 min and stored at -80°C until electrophoresis.

Supernatant was loaded on 13% potato starch gels dissolved in electrophoresis buffer (Cardy and Beversdorf, 1984) using three-stacked, 2 x 11 mm Whatman #2 filter-paper wicks. Electrophoresis was conducted at 6 °C, and 9.5 W 500 mL⁻¹ of constant current for 5 to 6 h to allow adequate separation of the aconitase isozyme bands.

After electrophoresis, gels were sliced horizontally into three slices. Both the top and the bottom slices were discarded. Aconitase isozyme activity was visualized by incubating gel slices at 37 °C for 2 h in a solution of 100 mL 0.2 M Tris buffer (pH 8), 200 mg cis-aconitic acid (pH 7), 100 mg magnesium chloride, 40 activity units isocitrate dehydrogenase, 7.5 mg β-nicotinamide adenine dinucleotide phosphate, 4 mg phenazine methosulfate (PMS) and 7.5 mg of thiazoyl blue tetrazolium bromide (MTT). The stain solution was mixed with 3 % agar dissolved in 20 mL of 200 mg cis-aconitic acid (pH 7), and applied as an overlay on the sliced gel. Each gel was screened to determine if there were any deviation from the expected Aco4-bb and Aco4-cc isozyme patterns.

Seedlings that expressed novel Aco4 isozyme alleles were transplanted into peat pots containing a standard soil mix (1 sand: 1 peat moss: 2 soil) in the USDA-ARS greenhouse for about a month. The plants were then transplanted in the field in a wide-
spaced HC design and allowed to self-pollinate in 2014. At harvest maturity, three 3-seeded pods were collected from each entry for aconitase isozyme electrophoresis as previously described to determine stability and heritability of the alleles.

**DNA analysis of the variants**

**Leaf sample collection and DNA extraction**

Leaf tissue from seedlings was sampled prior to being transplanted to the field. To avoid cross-contamination, a single leaf was inserted inside a 2 mL ziplock bag and the edge of the zip was used to cut the leaf sample, during the sealing of the ziplock bag. From seeds of pods collected in 2014, one seedling of each of the new *Aco4* isozyme variants (A13\(^v\)-bb-183-1 or A9\(^v\)-cc-229-3), and one seedling of the remaining two seeds from the same pod that contained the new variant (A13\(^v\)-bb-183-2 or A9\(^v\)-cc-229-2) were sampled. Also sampled was a new variant (A13\(^v\)-bb-67-1) detected in 2015 and one seedling of original ‘Jack’ cultivar (A182-cc) (Table 1). Leaf samples were immediately frozen at -80°C until DNA extraction. DNA was extracted using the CTAB method described by Saghai-Marooif et al. (1984). The extracted DNA was dissolved in 50 µL of ddH\(_2\)O. DNA quantity and quality were measured using a ND-1000 spectrophotometer (NanoDrop, Fisher Thermo, Wilmington, DE, USA), before storing at -20 °C until polymerase chain reaction (PCR) was conducted.

**PCR**

Nine forward and nine reverse primers (Integrated DNA technologies (IDT), Coralville, IA, USA) (Table 5) were used for the 25 µL PCR reactions. The reaction was comprised of 6 ng DNA per sample, 12.5 µL of 2X GoTaq Green Master Mix (Promega Corporation, Madison, WI), 1 µL of 5µM of each primer and 8.5µL RNase-free water.
DNA amplification was performed using a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Waltham, MA, USA) at the following conditions: temperature of 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 60 °C to 51 °C for 30 s, 72 °C for 1 min; then 25 cycles of 94 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. The PCR products were validated by loading 5 µL on a 1 % agarose-TBE gel, stained with 1 µg ml⁻¹ SYBR safe (Life Technologies Corporation, Carlsbad, CA, USA) in 1X TBE at 70 V for 80 min. Bio-Rad Gel Doc XR+ Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to capture the band pattern images from the gel. The product size was estimated by referencing the bands to a 0.9 µg 1kb Plus ladder (Life Technologies Corporation, Carlsbad, CA, USA). The remaining PCR product was cleaned with 25 µL of 7.5 M ammonium acetate and 100 µL of 100% ethanol (EtOH) followed by 100 µL of 70% EtOH. The air-dried pellet was dissolved in 20 µL of ddH₂O. Quantity and quality of the cleaned PCR product was measured by ND-1000 spectrophotometer (NanoDrop, Fisher Thermo, Wilmington, DE, USA).

**DNA Sequencing, alignment and data analysis**

Sequencing of the cleaned PCR amplicon of each sample was performed with 9 forward and 9 reverse primers (Table 2) using standard Sanger sequencing at Iowa State University DNA facility (Ames, IA). Sequence data for all fragments and samples were aligned using Sequencher (Sequencher version 5.4.6, 2016) software licensed to Iowa State University to determine variation among the samples. Protein prediction was performed using FGENESH+ (Solovyev, 2007, and a protein-protein BLAST was conducted in the National Center for Biotechnology Information (NCBI).

**Results**

**Aconitase isozyme pattern of seeds from wide-spaced HC planting design**

Before planting the experiments in 2013, five randomly selected seeds from each entry were electrophoretically tested to confirm Aco4 isozyme patterns. All entries had the expected Aco4 isozyme patterns (data not shown).

In 2013, two new variants were identified with a double Aco4 isozyme allele switching occurring in one seed within a three-seeded pod from two entries. Entry A13⁻bb plant 183 seed number one (A13⁻bb-183-1) changed from Aco4⁻bb to Aco4⁻cc, and entry A9⁻cc plant 229 seed number three (A9⁻cc-229-3) changed from Aco4⁻cc to Aco4⁻bb out of 75 seeds per entry (Table 1). Double allele switching in A13⁻bb-183-1 was a reversion from Aco4⁻bb back to the original Aco4⁻cc. Aco4 isozyme pattern of the remaining two seeds of each pod and the rest of the entries was as expected. These results suggested that the frequency of allele switching was low (~2.5%) when plants were exposed to a wide-spaced HC planting design.

These new variants were allowed to self-pollinate and three, 3-seeded pods were collected from each plant for Aco4 isozyme pattern analysis in 2014. The Aco4 isozyme patterns of the progenies of the new variants that switched from Aco4⁻bb to Aco4⁻cc remained Aco4⁻cc and the one that switched from Aco4⁻cc to Aco4⁻bb remained Aco4⁻bb indicating stability and heritability of the new alleles (Table 1).
In 2015, we identified one seed with a single allele switching from \( Aco4\text{-}bb \) to \( Aco4\text{-}bc \) in entry \( A13^v\text{-}bb \) plant 67 seed number one (\( A13^v\text{-}bb\)-67-1) (Table 1). However, this new variant developed as an abnormal seedling. Therefore, DNA from this seedling was extracted for sequencing. The \( Aco4 \) isozyme pattern of the other two seeds within the same pod, and all the remaining entries were unchanged (data not shown).

**Aconitase isozyme pattern of seeds from the modified AA treatment**

A total of 324 seeds from the modified AA and control plots were tested for \( Aco4 \) isozyme patterns in 2013 and 2014. In both years, no variation in \( Aco4 \) isozyme pattern was detected from all entries (data not shown).

**The \( Aco4 \) gene (Glyma.11g080600) sequencing**

Single nucleotide polymorphisms (SNPs) were detected between samples that had \( Aco4\text{-}bb \) and \( Aco4\text{-}cc \) (Table 2). We observed that SNPs detected in the new variant \( A13^v\text{-}bb\)-183-1 that changed from \( Aco4\text{-}bb \) to \( Aco4\text{-}cc \) were the same as those found in \( A9^v\text{-}cc\)-229-2 that did not change and the ‘Jack’ original cultivar, \( A182\text{-}cc \). Similarly, SNPs identified in the new variant \( A9^v\text{-}cc\)-229-3 with the new aconitase form \( Aco4\text{-}bb \) were the same as those in \( A13^v\text{-}bb\)-183-2, which had not changed (Table 2). SNPs identified in \( A13^v\text{-}bb\)-67-1 that had \( Aco4\text{-}bc \) were heterozygous for the two \( Aco4 \) isozymes, \( Aco4\text{-}bb \) and \( Aco4\text{-}cc \) variants. A total of 13 SNPs and two deletions were located in the introns. The 5’ untranslated region (UTR) and 3’UTR had one SNP each. We detected three SNPs in the exons located at 858, 1718 and 5579 bp (in bold letter in Table 2). A nucleotide substitution of G for C at position 858 (designated as G858C) caused an amino acid substitution of arginine (R) for proline (P) (bold letters in Fig. 1). A G5579A caused an amino acid substitution of arginine (R) for lysine (K) (bold letters in
Fig. 1). A silent substitution, C1718T, did not result in an amino acid substitution. Both nucleotides resulted in histidine (H) in the protein sequence (underlined bold letters in Fig. 1). Only one protein sequence was predicted from the DNA sequences of our samples.

Discussion

The loss of genetic diversity in highly homozygous cultivars of soybean is attributed to inbreeding. However, there is increasing evidence of novel genetic and phenotypic variation in soybean cultivars in response to environmental stimuli (Roth et al., 1989; Fasoula and Boerma et al., 2005, 2007; Yates et al., 2001; Espinosa et al., 2015; Sandhu et al., 2016) and fluidity of the genome (McClintock, 1984; Ceccarelli et al., 1997; Rasmusson and Phillips, 1997). Using RFLP, Roth et al. (1989) found new alleles in root tissues of two soybean cultivars, ‘Minsoy’ and ‘Noir’ and their crosses derived from tissue culture. Interestingly, these alleles were present in other soybean cultivars. Recent studies by Espinosa et al. (2015) detected single and double Aco4 isozyme allele switching, which the authors attributed to a genetic response to environmental stimuli. Most variants evaluated were stable except a few that reverted back to the original Aco4 allele or to a different form. Two new variants for Aco4 isozyme experienced double allele switching in one seed of a three-seeded pod. The frequency of variation was 1 out of 75 seeds in entry A13'-bb, in which both alleles switched to the original Aco4-cc from Aco4-bb, and 1 out of 75 seeds in entry A9'-cc, which switched from Aco4-cc to Aco4-bb. These new variants were stable and heritable as indicated by Aco4 isozyme pattern from progeny of the self-pollinated original variants
in 2014 (Table 1). Additionally, a single allele switching was detected from Aco4-\(bb\) to Aco4-\(bc\) in which the ‘c’ was an unexpected single allele reversion to the original form. The segregation pattern of this new single allelic variant was not followed because the seedling was abnormal and was only sampled for DNA analysis. However, based on prior studies with similar variants (Espinosa et al., 2015), it is likely that the variant could have segregated in a normal Mendelian manner. Environmental-stimuli-induced variants could explain a self-pollinated crop’s mechanism of adaptation to the ever-changing growing environment (Roth et al., 1989; Hopkins et al., 2013). All the new variants in our study were detected from plants grown in a wide-spaced HC planting design and not from plants whose seeds were exposed to a modified AA. Our results suggested that most of the variation observed by Espinosa et al. (2015) was the result of wide-spaced planting, and not the result of hail or exposing seeds to a modified AA before planting. In the wide-spaced HC planting design, plants are grown in wide- and equidistantly-spaced pattern thereby reducing interplant competition for growth resources (Fasoula and Fasoula, 1997). Since soybean is a row crop, it is most likely that the conditions under the wide-spaced HC planting design could create stimuli on individual plants and enhance expression of novel genetic and phenotypic variation, as plants adapt to these conditions. Previous studies observed genotypic and phenotypic variation in soybean cultivars selected from wide-spaced HC designs, which resulted in the release of new biotypes (Fasoula and Boerma, 2005, 2007; Fasoula et al., 2007a, 2007b, 2007c; Yates et al., 2009). Our study demonstrated that soybean variants are not permanent, but can revert back to the original genotypes, or are continuously generating intra-cultivar, genetic variation when exposed to environmental stimuli. However, the genetic variation
resulting from the response to environmental stimuli could be ignored due to the lower frequency of expression observed in our study and others (Amberger et al., 1992a, 1992b; Hopkins et al., 2013; Espinosa et al., 2015).

Our results cannot be explained based on mislabeling of samples or cross-pollination because samples were processed and tested in sequence. The double and single Aco4 isozyme allele switching were detected in a single seed within three-seeded pods collected from a wide-spaced HC design. Even though a single allele switch could be the result of an outcross, a double allele switch resulting from outcross is impossible. Moreover, we observed a single allele-switching rate of 2.8% at an interplant distance of 2.8 m. Ray et al. (2003) reported soybean cross-pollination rates of 0.003 % at 5.4 m and 0.41 % at 0.9 m from the pollen source, which indicated an inverse relationship between cross-pollination distance (Mercier et al., 2008). If cross-pollination was the source of the allele switching observed in our study, we would have expected a greater allele-switching percentage in seeds obtained from the modified AA plots, where variants with different Aco4 isozymes were planted next to each other in conventional rows at 0.76m between rows and 0.10 m between plants. The expected rate of natural cross-pollination between soybean plants in conventional rows was reported to range from 0.004% to 2.5% (Weber and Fehr, 1967; Ahrent and Caviness, 1994). However, no genetic changes were detected in three-seeded pods collected from the conventional-row planting. Furthermore, the occurrence of a single seed with a single allele switch suggests that both cross- and self-pollination occurred in the same flower. To our knowledge, Espinosa et al. (2015) were the only researchers to report finding one soybean pod out of 384 pods containing one hybrid and two self-pollinated homozygous seeds for the Aco4 locus in naturally
pollinated flowers. The authors attributed these results to new genetic variation. We also observed double alleles switching in one seed within a pod. This never reported before event cannot be explained based on cross-pollination.

Molecular characterization of the new variants indicated SNP substitutions in the exons that resulted in amino acid substitution and slightly different proteins between Aco4-bb and Aco4-cc samples (Fig. 1). Coleman et al. (2016) also found that SNP substitutions in the exon of the Aco4 gene in two soybean cultivars resulted in amino acid changes and different protein sequences. The authors hypothesized that these differences could produce different protein structures and aconitase isozyme patterns. The seed used in their study was collected from ultra-low plant density. Brettell et al. (1986) also reported finding one alcohol dehydrogenase (Adhl) variant from 645 maize tissue culture plants. Sequencing of the Adhl gene indicated that a SNP substitution in the exon resulted in an amino acid change from valine to glutamic acid, resulting in a different protein structure with a different mobility pattern. Our results showed that the aconitase allele switching was associated with genetic changes at the Aco4 locus.

Another explanation for the Aco4 isozyme allele switching could be alternative splicing (AS), which results in different proteins forms with different sequences, stability and localization (Kazan, 2003; Staiger and Brown, 2013). This can be accomplished by a mutation in the splice sites or splice signals that create immature stop codons resulting in truncated proteins (Shen et al., 2014). One example is the Waxy (Wx) gene responsible for grain amylose content in rice. A substitution of guanosine for uridine at the 5’ splice site of an intron induced two hidden splice sites in an exon that negatively affected normal splicing (Cai et al., 1998; Larkin and Park, 1999). This abnormal splicing resulted
in reduced amylose levels in rice seed. In our study, the identified SNPs were not located at any of the splice sites and only one protein sequence was predicted from the observed SNPs. Therefore, the observed Aco4 isozyme differences cannot be attributed to AS.

Transposable elements also may cause insertions or deletions in genomic sequences (Palmer 1984; Caspi and Pachter, 2006). For instance, the chlorophyll-deficient malate-dehydrogenase null variant in soybean resulted from a chromosomal deletion caused by a transposable element (Palmer, 1984). However, our sequence data alignments did not show any significant deletions or insertions that could explain the observed Aco4 isozyme differences.

Genetic variation in near homozygous cultivars also has been attributed to pre-existing variation in parental plants that may be hidden for several generations but reappear when plants are exposed to environmental stimuli (Haun et al., 2011; Yates et al., 2012). For example, using SSR markers, Yates et al. (2012) found that most of the phenotypic variation identified in 19 lines selected from three soybean cultivars planted at ultra-low density was pre-existing in original parents (Yates et al., 2012). However, some variation could not be traced back to original parents. This variation was attributed to newly induced genetic variation. Similar results were found in ‘Williams 82’, where almost all observed genetic variation was residual (Haun et al. (2011). In our study, we evaluated genetic variation at the Aco4 gene in foundation seed of ‘Jack’ cultivar. However, we did not detect any genetic changes, which could be attributed to pre-existing variation.

The response mechanism of Aco4 allele switching observed in our study may be mediated by iron availability in plant cells. Aconitase is comprised of a three iron-sulfur
cluster (3Fe-4S) in its inactive form, and is activated through the binding of a fourth free iron to form a 4Fe-4S cluster (Zhou and Ragan 1995; Gardner 1997; Shlizerman et al., 2007). Studies have shown that inadequate iron in plant cells may reduce aconitase isozyme activity (McCluskey et al. 2004; Shlizerman et al., 2007) resulting in stunted plant growth (Carrari et al., 2003). Interestingly, we observed a stunted growth phenotype in Aco4-cc variants, including the revertant that switched from Aco4-bb to Aco4-cc in A13-bb-229 (data not shown). The plants matured earlier compared with those with Aco4-bb. These observations suggest that these genetic changes could be influencing plant metabolism. The phenotypic observations also could indicate soybean genome-wide changes. In our study, we observed variability among entries within the variants for maturity, plant height, seed weight, seed yield and seed composition compared with ‘Jack’ original cultivar (data not shown). Further studies on the role of iron-sulfur cluster and genome-wide changes occurring in these variants could shed light on the mechanisms of these aconitase isozyme allele switching.

Conclusion

This study evaluated stability of the Aco4 isozymes in soybean ‘Jack’ variants seeds from plants grown in a wide-spaced HC planting design and in seeds from plants whose seed was exposed to a modified AA before planting. Also, we determined genetic differences between variants and ‘Jack’ original foundation seed at the Aco4 gene, Glyma.11g080600. Our results showed that genetic variation in homozygous cultivars exposed to environmental stimuli is a continuous process, and variants are not a
permanent record but can revert to the original genotype or a to different genotype in response to environmental stimuli. We identified nucleotide substitutions (SNPs) in the exons from our sequence data that resulted in amino acid substitution and slightly different proteins between $Aco4-bb$ and $Aco4-cc$ samples. This SNPs difference could explain the differences observed in the $Aco4$ isozyme mobility patterns. In our study, aconitase allele switching was caused by genetic changes. However, genome-wide changes and properties of the aconitase iron-sulfur cluster in these variants need to be studied to assess their influence in the aconitase isozyme allele switching. Our study showed that the wide-spaced HC planting design may be a valuable tool for generating intra-cultivar genetic variation. Plant breeders could use wide-spaced HC planting design to induce novel genetic variation for cultivar development.

**Acknowledgements**

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References


Table 1. Seed source for cultivar ‘Jack’ Aconitase 4 (Aco4) isozyme experiment from 2008 to 2015.

<table>
<thead>
<tr>
<th>Year</th>
<th>Planting pattern</th>
<th>Number of plants (Entries)†</th>
<th>Entry with Aco4 allele switching‡</th>
<th>Number of seeds tested§</th>
<th>Aco4 isozyme pattern of planted seed</th>
<th>Aco4 isozyme pattern of harvested 3-seeded pods¶</th>
<th>Frequency of Aco4 variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>Honeycomb (HC)</td>
<td>500</td>
<td></td>
<td>1500</td>
<td>Aco4-cc (‘Jack’)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>Accelerated Aging plots (AA); 1152 seeds</td>
<td>128</td>
<td>A127-cc</td>
<td>1152</td>
<td>Aco4-cc</td>
<td>Aco4-bc</td>
<td>1/1152</td>
</tr>
<tr>
<td>2010-2011</td>
<td>Stability of Aco4-bc in rows</td>
<td>50</td>
<td>A127-cc</td>
<td>150</td>
<td>Aco4-bc</td>
<td>Aco4-bc, Aco4-bb</td>
<td>4/1152</td>
</tr>
<tr>
<td>2012</td>
<td>Self-pollination and seed increase of variants and original ‘Jack’</td>
<td>50</td>
<td></td>
<td>150</td>
<td>Aco4-bb</td>
<td>Aco4-bb</td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>HC</td>
<td>25</td>
<td>A13*-bb-183</td>
<td>75</td>
<td>Aco4-bb</td>
<td>Aco4-bb</td>
<td>1/75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>A9*-cc-229</td>
<td>75</td>
<td>Aco4-bb</td>
<td>Aco4-bb</td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>HC (transplants)</td>
<td>1</td>
<td>A13*-bb-183</td>
<td>9</td>
<td>Aco4-bb</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>A9*-cc-229</td>
<td>9</td>
<td>Aco4-bb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2015</td>
<td>HC</td>
<td>12</td>
<td>A13*-bb-67</td>
<td>36</td>
<td>Aco4-bb</td>
<td>Aco4-bc</td>
<td>1/36</td>
</tr>
</tbody>
</table>

†Number of single plants or entries planted in wide-spaced honeycomb planting design or conventional rows from which three-seeded pods were collected for aconitase isozyme test.
‡Entries that had single or double Aco4 allele switching in one seed of the three-seeded pods. Nomenclature used is as follows: gene (A=aconitase), entry number, type [original or variant (') (Espinosa et al., 2015)], allelic pattern (bb or cc) and plant number assigned in 2013. All other entries in the study had the expected Aco4 isozyme pattern.
§Total number of seeds from three-seeded pods tested for Aco4 allele switching per entry.
¶Samples with the Aco4 isozyme pattern in bold were sequenced. These samples included one seedling of each of the new Aco4 isozyme variant, one seedling of the remaining two seeds in a pod of each of the new variants, 2015 new variant and one seedling of original ‘Jack’ cultivar. The 2013 new variants were self-pollinated in 2014. The 2015 new variant was abnormal; therefore the whole plant was sampled for sequencing.
Table 2. Forward and reverse primer, PCR product size, start and end position of the primer pairs used for PCR and sequencing *Glyma.11g080600* of the new cultivar ‘Jack’ Aco4 variants and original ‘Jack’ cultivar generated from wide-spaced honeycomb planting design.

<table>
<thead>
<tr>
<th>Primer†</th>
<th>Primer forward sequence</th>
<th>Primer reverse sequence</th>
<th>PCR product size (bp)</th>
<th>Start position</th>
<th>End position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F: ATGGCGTGCGATATTTCCTT</td>
<td>R: GCTACATTACATTTCCCCGAAAA</td>
<td>927</td>
<td>6058520</td>
<td>6059446</td>
</tr>
<tr>
<td>2</td>
<td>F: TTTTCGGGGAATGTAAATGAGC</td>
<td>R: TCAGGATAAAGCACACCATTTTGT</td>
<td>987</td>
<td>6059425</td>
<td>6060411</td>
</tr>
<tr>
<td>3</td>
<td>F: CTTTATCCTGACACGTTGTTG</td>
<td>R: GGACCTGAGACACAGGTTCTTA</td>
<td>992</td>
<td>6060401</td>
<td>6061392</td>
</tr>
<tr>
<td>4</td>
<td>F: TGTAGAACCTGTCTCAGGTC</td>
<td>R: CTACTGTACCCTTCTGCGCTTG</td>
<td>833</td>
<td>6061370</td>
<td>6062201</td>
</tr>
<tr>
<td>5</td>
<td>F: CAAGGCCAGAAGGATACAGTAG</td>
<td>R: AGAAATGGGAAAAAGCAACAGAA</td>
<td>1025</td>
<td>6062180</td>
<td>6063204</td>
</tr>
<tr>
<td>6</td>
<td>F: CAAGCATGATCTGGAAGGTTACA</td>
<td>R: GAAGATCTTTGTGGACATTCTTC</td>
<td>944</td>
<td>6063087</td>
<td>6064009</td>
</tr>
<tr>
<td>7</td>
<td>F: GCATTTCAAAACCTTGCTGTCAC</td>
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<tr>
<td>8</td>
<td>F: CTGTCTTTAAGTGGCACTCTTTA</td>
<td>R: ACGCCCTAGAAAACAATAACGAA</td>
<td>999</td>
<td>6064895</td>
<td>6065872</td>
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<tr>
<td>9</td>
<td>F: TGGATCCTTTAAGTGGCATTGTTCT</td>
<td>R: ATTTTTCTCCTTCCGCATTTC</td>
<td>909</td>
<td>6065864</td>
<td>6066772</td>
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†Nine primer pairs were used to sequence the *Glyma.11g080600*
Table 3. Single nucleotide polymorphisms (SNPs) identified among six soybean entries/variants and their location on Glyma.11g080600 gene sequenced in 2016.

<table>
<thead>
<tr>
<th>Entry†</th>
<th>Aco4 pattern before HC</th>
<th>Aco4 pattern after HC</th>
<th>Location / SNP‡</th>
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<tr>
<td></td>
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<tr>
<td>A13⁻⁻⁻⁻⁻⁻-bb-67-1</td>
<td>bb</td>
<td>bc</td>
<td>AT</td>
</tr>
<tr>
<td>A13⁻⁻⁻⁻⁻⁻-bb-183-1</td>
<td>bb</td>
<td>cc</td>
<td>A</td>
</tr>
<tr>
<td>A13⁻⁻⁻⁻⁻⁻-bb-183-2</td>
<td>bb</td>
<td>bb</td>
<td>T</td>
</tr>
<tr>
<td>A9⁻⁻⁻⁻⁻⁻-cc-229-2</td>
<td>cc</td>
<td>cc</td>
<td>A</td>
</tr>
<tr>
<td>A9⁻⁻⁻⁻⁻⁻-cc-229-3</td>
<td>cc</td>
<td>bb</td>
<td>T</td>
</tr>
<tr>
<td>A182-cc</td>
<td>cc</td>
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(Continued below)

Table 3. Continued

<table>
<thead>
<tr>
<th>Entry†</th>
<th>Aco4 pattern before HC</th>
<th>Aco4 pattern after HC</th>
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<tr>
<td></td>
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<td>5579</td>
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<tr>
<td>A13⁻⁻⁻⁻⁻⁻-bb-67-1</td>
<td>bb⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>bc⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>AG</td>
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<tr>
<td>A13⁻⁻⁻⁻⁻⁻-bb-183-1</td>
<td>bb⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>cc⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>G</td>
</tr>
<tr>
<td>A13⁻⁻⁻⁻⁻⁻-bb-183-2</td>
<td>bb⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>bb⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>A</td>
</tr>
<tr>
<td>A9⁻⁻⁻⁻⁻⁻-cc-229-2</td>
<td>cc⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>cc⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>G</td>
</tr>
<tr>
<td>A9⁻⁻⁻⁻⁻⁻-cc-229-3</td>
<td>cc⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>bb⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>A</td>
</tr>
<tr>
<td>A182-cc</td>
<td>cc⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>cc⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>G</td>
</tr>
</tbody>
</table>

†Entries highlighted with the same color have similar SNPs in all locations on the gene. The entries that were sequenced included one seedling of each of the new Aco4 isozyme variant (A13⁻⁻⁻⁻⁻⁻-bb-183-1 and A9⁻⁻⁻⁻⁻⁻-cc-229-3), one seedling of the remaining two seeds in a pod that contained the new variant (A13⁻⁻⁻⁻⁻⁻-bb-183-2 and A9⁻⁻⁻⁻⁻⁻-cc-229-2), 2015 new variant (A13⁻⁻⁻⁻⁻⁻-bb-67-1) and original ‘Jack’ cultivar (A182-cc).

‡SNPs in bold are located in exons. SNPs at 236 and 8150 are located in the 5’UTR and 3’UTR respectively. Positions 1493, 1494, 5684 and 5687 indicate deletions in the introns. The rest of the SNPs are located in the introns.
**Aco4-bb** MATENPFNSILTLLEKGGAGEFGKYFSLPALNDPRIDRLPSYRILLESAINRCDEFQV 60
**Aco4-cc** MATENPFNSILTLLEKGGAGEFGKYFSLPALNDPRIDRLPSYRILLESAINRCDEFQV 60

******************************************************************************
**Aco4-bb** KSNDEVKIDWENTSPLKEIFPKPARVLLQDFGTPAVPVDVLACMRDAMKLGDSDKIN 120
**Aco4-cc** KSNDEVKIDWENTSPLKEIFPKPARVLLQDFGTPAVPVDVLACMRDAMKLGDSDKIN 120

******************************************************************************
**Aco4-bb** PLVPDLVLDHSVQDVARSENAVQANMELEFQRNKRFGFLKGNSAFNNMLVPPGSG 180
**Aco4-cc** PLVPDLVLDHSVQDVARSENAVQANMELEFQRNKRFGFLKGNSAFNNMLVPPGSG 180

******************************************************************************
**Aco4-bb** IV_QVNLEYLGRVFNONGLYPSVGVTDSTHTMDIQLGVAGWGVEISEAEAMLGQPM 240
**Aco4-cc** IV_QVNLEYLGRVFNONGLYPSVGVTDSTHTMDIQLGVAGWGVEISEAEAMLGQPM 240

******************************************************************************
**Aco4-bb** SMVLPVGVFKLLGLRDLGVTATDLVTQLMLRHKGVGFKEVPFVEFGSEGSMSLADAR 300
**Aco4-cc** SMVLPVGVFKLLGLRDLGVTATDLVTQLMLRHKGVGFKEVPFVEFGSEGSMSLADAR 300

******************************************************************************
**Aco4-bb** IANMSPEYGMTGFPFPDHTQLYRLTRGSRTSDETVSIESYLRANKMKFVDYSEPQVERVY 360
**Aco4-cc** IANMSPEYGMTGFPFPDHTQLYRLTRGSRTSDETVSIESYLRANKMKFVDYSEPQVERVY 360

******************************************************************************
**Aco4-bb** SSYLELNLEDVEPCVSFKRHPDRVPLREMVKVHLAACLNNKGFKGFAVPQNKVAEF 420
**Aco4-cc** SSYLELNLEDVEPCVSFKRHPDRVPLREMVKVHLAACLNNKGFKGFAVPQNKVAEF 420

******************************************************************************
**Aco4-bb** TFQGTPFAHRHGVDVIAATSCNTSNPSVNLGALVAKKACELQGKPVKITSALPGS 480
**Aco4-cc** TFQGTPFAHRHGVDVIAATSCNTSNPSVNLGALVAKKACELQGKPVKITSALPGS 480

******************************************************************************
**Aco4-bb** GVTYKLQSGTQKYNLEGFPNIVGCTCTCIGNSGDINEAVASAITENDIAVAAVLGN 540
**Aco4-cc** GVTYKLQSGTQKYNLEGFPNIVGCTCTCIGNSGDINEAVASAITENDIAVAAVLGN 540

******************************************************************************
**Aco4-bb** RNFEVRHPTRANYLSPPLVAYALAGTVDIDFTDEPFIGKGTKFKDIWSSSE 600
**Aco4-cc** RNFEVRHPTRANYLSPPLVAYALAGTVDIDFTDEPFIGKGTKFKDIWSSSE 600

**Figure 1.** Alignment of protein sequences from *Aco4*-bb and *Aco4*-cc samples. All sequenced samples that had the same *Aco4* isozyme pattern had similar SNPs and protein sequences. Bold letters indicate different amino acids between the two samples produced from SNP changes in the exons. Underlined bold letters indicate the same amino acid produced from a silent SNP change in the exon. Samples with *Aco4*-bb were A13v-bb-183-2 and A9v-cc-229-3, and those with *Aco4*-cc were A13r-bb-183-1, A9v-cc-229-2 and A182-cc.
<table>
<thead>
<tr>
<th>Variant</th>
<th>Amino Acid Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aco4-bb</td>
<td>IANVVQSSVLPMFRDTRYNAITQGNPMWNNLSVPTGTLYAWDPTSTYIHHEPPYFRDMSMS</td>
<td>660</td>
</tr>
<tr>
<td>Aco4-cc</td>
<td>IANVVQSSVLPMFRDTRYNAITQGNPMWNNLSVPTGTLYAWDPTSTYIHHEPPYFRDMSMS</td>
<td>660</td>
</tr>
<tr>
<td>Aco4-bb</td>
<td>PPGSHGVKDAYCLLNFGDSITTDHISPAGSIIKDSPAARYLIERGVDRRDFNSYGSRRGN</td>
<td>720</td>
</tr>
<tr>
<td>Aco4-cc</td>
<td>PPGSHGVKDAYCLLNFGDSITTDHISPAGSIIKDSPAARYLIERGVDRRDFNSYGSRRGN</td>
<td>720</td>
</tr>
<tr>
<td>Aco4-bb</td>
<td>DEVMARGTFANIRIVNKFLNGEVGPKTIHIPSGEKLSVFDAAEKYKSEGHMILAGAEY</td>
<td>780</td>
</tr>
<tr>
<td>Aco4-cc</td>
<td>DEVMARGTFANIRIVNKFLNGEVGPKTIHIPSGEKLSVFDAAEKYKSEGHMILAGAEY</td>
<td>780</td>
</tr>
<tr>
<td>Aco4-bb</td>
<td>GSGSSRDWAAGPMLLGVKAVIAKSFERIHRSNLVGMIGIPLCFKPDDADSLGTLGHER</td>
<td>840</td>
</tr>
<tr>
<td>Aco4-cc</td>
<td>GSGSSRDWAAGPMLLGVKAVIAKSFERIHRSNLVGMIGIPLCFKPDDADSLGTLGHER</td>
<td>840</td>
</tr>
<tr>
<td>Aco4-bb</td>
<td>YTIDLPSNVNEIRPGQDTVVTADGSFVSTLRFDTEVELAYFNHGILQYVIRNMVNAK</td>
<td>900</td>
</tr>
<tr>
<td>Aco4-cc</td>
<td>YTIDLPSNVNEIRPGQDTVVTADGSFVSTLRFDTEVELAYFNHGILQYVIRNMVNAK</td>
<td>900</td>
</tr>
</tbody>
</table>

Figure 1. Continued
Figure 2. Seed source for the stability of aconitase isozyme in ‘Jack’ variants experiment from 2013 to 2016. A solid bold line indicates the initiation of this experiment (2013).
CHAPTER 5. GENERAL CONCLUSIONS

The main objectives of this research were to evaluate agronomic performance, seed characteristics and aconitase isozyme variability, and stability of soybean cultivars and variants in response to environmental stimuli. The stimuli included the wide-spaced honeycomb (HC) planting design, simulated hail and a modified accelerated aging (AA). These studies were undertaken to separate the effect of a single environmental stimulus from that of multiple environmental stimuli on genotypic and phenotypic variation of soybean cultivars. If successful, a single environmental stimulus could be used to generate novel variation for cultivar development.

The first study evaluated agronomic performance and seed characteristics in progenies of ‘BSR 101’ and ‘Jack’ soybean cultivars grown in wide-spaced HC planting design and conventional row (CR) planting with and without simulated hail. Our results showed that plant height, maturity and seed weight of progenies from plants planted in a wide-spaced HC planting design were significantly different than those from CR planting. Progenies from plants without simulated hail in wide-spaced HC planting design and CR planting had greater seed yield than those with simulated hail. Protein content was higher in progeny from plants in CR planting with simulated hail than those without simulated hail. Simulated hail treatment did not have a significant effect on agronomic performance and seed characteristics. Prior research showed agronomic variation and new biotypes (Espinosa et al., 2015; Sandhu et al., 2016) in progeny from plants in a wide-spaced HC planting design exposed to hail. However, the effects of planting distance and hail could not be separated. The present study identified the effect
of individual environmental stimuli of planting distance and simulated hail on the agronomic performance, seed characteristics, and intra-cultivar variation of progeny from the affected plants. These results showed a significant agronomic performance variation in progenies from homozygous cultivars exposed to different environmental stimuli. This method for generating new biotypes could be useful to plant breeders. These findings also could be important to soybean seed producers. When soybean seed production fields are affected by a hailstorm during the plant’s vegetative growth, the progeny from these affected plants may show unexpected agronomic variation. Future studies should assess the expression and stability of superior traits in progenies from wide-spaced HC planting design for multiple generations.

The second study evaluated agronomic performance, seed characteristics and variability in progenies from entries of ‘Jack’ variant\(^b\), ‘Jack’ variant\(^c\), and ‘Jack’ original cultivar planted in a wide-spaced HC planting design or exposed to a modified AA at planting. The study was initiated to understand the effect of planting distance or artificial aging environmental stimuli on the agronomic performance and seed characteristics stability of variants. The results showed that agronomic performance and seed characteristics of progeny from entries within ‘Jack’ variant\(^b\) and ‘Jack’ variant\(^c\) were more variable than those in ‘Jack’ original cultivar for almost all traits evaluated. These variants had higher yield, oil content, shorter or longer maturity, and other agronomic changes and seed characteristics. Agronomic performance differences in progenies of cultivars exposed to environmental stimuli can be substantial and could be useful when breeding for new cultivars with improved agronomic performance and seed
characteristics for improved food security. Further research into the stability of these traits over generations will determine their usefulness as new sources of germplasm.

The third study evaluated stability of the Aco4 isozymes in soybean seeds of ‘Jack’ variants from plants grown in a wide-spaced HC planting design and plants from seeds exposed to modified AA before planting. The stability of these variants over generations and after additional environmental stimuli is critical for utilizing these variants in plant breeding. Also, we determined genetic differences between variants and ‘Jack’ original foundation seed at the Aco4 gene, Glyma.11g080600 and elucidated the genetic mechanisms behind aconitase allele switching. The results showed that genetic variation in soybean cultivars exposed to environmental stimuli is a continuous process; and variants are not a permanent record, but can revert back to the original genotype or a different genotype to adapt to the stimuli. We identified SNPs substitutions in the exons from our sequence data that resulted in amino acid substitution and slightly different proteins between Aco4-bb and Aco4-cc samples. This SNPs difference could explain the differences observed in the Aco4 isozyme mobility patterns. These results indicated that the aconitase allele switching was caused by genetic changes. However, genome-wide changes, and properties of the aconitase iron-sulfur cluster need to be investigated for understanding their role in aconitase allele switching of these variants. This research confirmed that a wide-spaced HC planting design is a valuable tool for generating novel genetic variation. Plant breeders could use wide-spaced HC planting design as a tool to induce useful novel genetic variation for cultivar development.
References
