

Insect-mediated cross-pollination in soybean [*Glycine max* (L.) Merrill]

I. Agronomic performance[☆]

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Abstract

In soybean, manual cross-pollination to produce large quantities of F₁ hybrid seed for yield trials is difficult and time-consuming. Conversely, insect-mediated cross-pollination has been shown to produce large quantities of hybrid seed in soybean and could facilitate the identification of heterotic patterns. The objective of our study was: (1) evaluate F₁ hybrid soybean plants from single crosses for yield and agronomic traits over several environments and (2) compare hybrid performance of the single crosses to lines developed from three-way crosses and backcrosses. In 2003, F₁ seed of single-crosses and their parent lines were evaluated in replicated experiments at three locations. Also in 2003, three-way crosses, and BC₁F₁ seed were produced. In 2004, three-way crosses, BC₁F₁ crosses, and their parent lines were evaluated at one location. High-parent heterosis (HPH) in single-crosses for grain yield ranged from −41.11% to +11.19%; for protein content from −4.34% to +3.53%, and for oil content from −13.22% to −0.84%. In three-way crosses, HPH for yield ranged from −25.21% to −4.50%, for protein from −2.72% to +1.92%, and for oil from −5.87% to −1.20%. For BC₁F₁ crosses, HPH for yield ranged from −15.65% to +41.97%, for protein from −2.57% to +1.69%, and for oil from −2.47% to +2.22%. Although positive heterosis levels were observed across all populations tested to determine the economic feasibility it is imperative that more tests of more cross-combinations be evaluated in replicated environments. Extensive research in different environments must be conducted to determine what parental combinations will produce the highest heterosis levels, and to develop criteria for selecting the parents with the best combining ability. This will be important to maximize agronomic performance that can economically justify the use of hybrids in soybean production.

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1. Introduction

The phenomenon of heterosis or hybrid vigor (Falconer and Mackay, 1996) has been studied extensively in allogamous and other plant species with mixed reproductive systems, i.e., maize

[*Zea mays* (L.)], sorghum [(*Sorghum bicolor* (L.) Moench)], pearl millet [(*Pennisetum glaucum* (L.) R. Br.], rapeseed [*Brassica napus* (L.)], onion [*Allium cepa* (L.)], sunflower [*Helianthus annuus* (L.)], cotton [*Gossypium hirsutum* (L.)], and tomato (*Lycopersicon esculentum* Mill.). Heterosis is commercially exploited in seed production, where F₁ hybrids have shown to considerably increase seed yield. Additionally, the use of hybrids has other benefits such as the possibility of developing genotypes of earlier maturity, uniformity of the harvested products, and the stacking of several other useful traits including disease and herbicide resistance (Palmer et al., 2003). Important related issues added to these benefits are the intellectual property-right system in hybrid commercialization

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and protection of parental inbreds used in hybrid production. These latter issues allow private seed companies to legally protect their products and parentage of hybrids (Wehner, 1999).

In spite of these advantages, the use of hybrids has some limitations. For instance, in some of the self-pollinated crops, hybrids for commercial plantings are considered impractical because of the strict mechanisms of self-pollination that greatly reduces cross-pollination (Palmer et al., 2001). This is the case for rice [*Oriza sativa* (L.)], wheat [*Triticum* spp. (L.)], tobacco [*Nicotiana tabacum* (L.)], and soybean [*Glycine max* (L.) Merr.]. However, in recent years success has been achieved in the commercial use of hybrid rice (Virmani, 1997, 1999), and in the development of hybrid wheat (Jordaan et al., 1999), and pigeon pea [*Cajanus cajan* (L.) Millsp.], (Plant Breeding News IV, ed. 107, Nov, 1999). In soybean, use of hybrid seed for commercial production is not yet a reality. In spite of difficulties in manually producing hybrid seed in soybean, interest in its use for commercial production has remained high and studies have been conducted to determine heterosis values for traits of economic importance. Average mid-parent yield heterosis determined for 2, 8, 27, 18, and 18 hybrid combinations were 28, 13, 8, 2, and 8%, respectively (Brim and Cockerham, 1961; Hillsman and Carter, 1981; Nelson and Bernard, 1984) suggesting that it is possible to find parent combinations that could give a significant economic increase to seed production. These results, however, cannot be extrapolated to commercial production fields since estimates were collected with spaced F₁ plants. To evaluate hybrid combinations on a large scale and to obtain meaningful results about heterosis in soybean, large amounts of hybrid seed need to be produced.

In soybean, naturally occurring mutations that impair male function have been found, identified, and their inheritance studied (Graybosch and Palmer, 1988). Use of these male-sterile lines to produce hybrid seed would require that the male-sterile female-fertile system be biologically stable, and that an efficient mechanism of pollen transfer from male to female parent be found (Palmer et al., 2001). However, in soybean, male sterility has been used primarily to generate random mating populations, or to facilitate hybridization in pure-line development programs (Specht and Graef, 1992; St. Martin and Ehounou, 1989; Carter et al., 1983). Despite the fact that soybean is a self-pollinated species, soybean flowers possess most of the floral characteristics of entomophilus plants (Juliano, 1976; Erickson and Garment, 1979; Arroyo, 1981; Erickson, 1983; Delaplane and Mayer, 2000; Horner et al., 2003), suggesting that insect vectors could be a practical means by which pollen can be transferred. Previous studies have shown that insect-mediated cross-pollination may be used to produce large quantities of hybrid seed, which along with selection practiced for insect attractiveness in male-sterile lines has made possible the production of hybrid seed on a larger scale than before. Thus, it will be possible to evaluate replicated row plot tests. Hybrid F₁ soybean seed production using insect-mediated cross-pollination presents several advantages over manual cross-pollination: (1) larger amounts of seed are produced on the male-sterile plants; (2) it is less time and labor

consuming; (3) it makes possible producing hybrid seed from a larger number of cross-combinations (which in turn would facilitate the identification of heterotic patterns); (4) conventional yield trials can be conducted with larger amounts of F₁ seed (the testing of spaced F₁ plants, single-row plots, short-length plots, etc., is avoided) (Lewers and Palmer, 1997; Lewers et al., 1996, 1998; Ortiz-Perez et al., 2004).

The objectives of our study were: (1) evaluate F₁ hybrid soybean plants from single-crosses for yield and agronomic traits over several environments, and (2) compare hybrid performance of the single-crosses to lines developed from three-way crosses and backcrosses using male-sterile female-fertile and male-fertile parents and bees from the families Megachilidae, Halictidae, Anthophoridae, and Andrenidae as insect pollen mediators.

2. Materials and methods

2.1. Plant materials

The female parents were soybean lines with nuclear male-sterile mutations. The selected female parents were male-sterile, female-fertile lines segregating for *ms2* in two different genetic backgrounds: (L75-0587) (Bernard et al., 1991), and *ms2* (A00-39 and A00-41) (Cervantes-Martinez et al., 2005); *ms3* (T284H) (Chaudhari and Davis, 1977); *ms6* (T295H) (Skorupska and Palmer, 1989); *ms8* (T358H) (Palmer, 2000); *ms9* (T359H) (Palmer, 2000). The female lines segregating for *ms3*, *ms6*, *ms8*, and *ms9* are non-allelic to each other, and phenotypic expression of male sterility was different for each line. These mutant female lines also showed differences for seed-set, thus, they were selected for high and low seed-set from a group of male-sterile lines previously evaluated in a three-year experiment at Ames, IA (Ortiz-Perez et al., 2006). The male parents were eight male-fertile, female-fertile lines chosen for attractiveness to pollinator insects and/or agronomic characteristics.

2.2. Single-cross hybrid seed production

Single-cross hybrid seeds were produced by using a randomized complete block design (RCBD) with five replications and eight entries. Each entry was the combination of one female parent and one male parent. Plots were six rows wide, with the first and the sixth planted with males and the four center rows were planted with the females. Each row was 4.8 m long, spaced 76 cm between rows and 1.2 m among plots, seeding rate was 14 seeds per meter. The eight single-combinations were cross-pollinated using alfalfa leaf cutter bees (*Megachile rotundata* F.) as pollinator in the summer of 2002 at Plainview, Texas. At stage R1 (Fehr et al., 1971), beginning flowering, alfalfa leaf cutter bee pupae were placed in a container on the base of a bee board, consisting of a wooden board with 10.6-cm by 10.6-cm wood cylinders, 1.2 m long, with closely spaced holes 0.47 cm in diameter, and 8.89 cm deep. Approximately 10,000 pupa were placed per bee board, and only one bee board was placed in the center of the

experimental plots. The bees started to emerge as adults in 2 days.

At flowering stage R2 (Fehr et al., 1971), female plants were identified by pollen abortion and were labeled. The male-fertile siblings tend to have earlier flowering compared to their male-sterile siblings, so they were rouged out at R1 (Fehr et al., 1971), usually before the female plants started flowering. Each female plant was checked three times during the flowering period, using at least 10 flowers/male-sterile plant. At maturity stage R8 (Fehr et al., 1971), the number of pods and seeds per female plant were recorded. The two lines segregating for *ms2* (A00-39 and A00-41) (Cervantes-Martinez et al., 2005), which are considered sister lines, were cross-pollinated to a commercial high-yielding line (DSR Exp. 202b) in November 2002 at Racagua, Chile, using the same insect pollinator species and procedures as in Texas (Fig. 1).

2.3. Three-way crosses and BC_1F_1 hybrid seed production

A sample of the F_1 seed obtained at Texas in 2002 was planted in the off-season nursery at Isabela, Puerto Rico, in October 2002 under natural photoperiod; and each F_2 plant was individually harvested. Fifty F_2 seeds from F_1 (*Msm*) plants with the most seed within each entry were selected for planting in the nursery in January 2003 under artificial light to extend the natural photoperiod, this consisted of exposure to continuous light for 15 days after emergence, to 14.5 days for an additional 35 days, and to natural day length thereafter. Only fertile F_2 (*Ms_*) plants were harvested and considered as a family. In the summer of 2003 at Plainview, Texas 24 $F_{2:3}$ derived families per

cross-combination were insect-mediated cross-pollinated to their male-fertile, female-fertile recurrent parents and also to a common parent (Fig. 1). Native bees from families Megachilidae, Halictidae, Anthophoridae, and Andrenidae were observed to carry out the pollinations.

Families were planted in a randomized complete block design (RCBD) with 6 replications and 16 entries to produce the hybrid seed. Each plot was six rows, the first and the sixth rows were males and the four center rows were the segregating male-sterile, female-fertile lines. Each row was 4.8 m long, spaced 76 cm between rows and 1.2 m between plots, the planting rate was 14 seeds per meter. Each entry was the combination of one segregating male-sterile, female-fertile line and one male-fertile, female-fertile line. At flowering, male-sterile plants were identified and labeled, and male-fertile siblings removed as previously described. At maturity, the number of pods and number of seeds per male-sterile plant were recorded.

2.4. Field trials

2.4.1. Single-crosses

Each single-cross combination F_1 hybrid seed produced in Texas and Chile was bulked. The cross A00-73 *ms9* × Raiden was not included in the field trials in 2003 because only a few female plants could be harvested; however, F_1 seed from those plants were used to produce the three-way and BC_1F_1 seeds. All other F_1 hybrid seed of the single-crosses, parents, and three commercial lines used as checks, were grown at the Bruner Farm near Ames, IA (42°03'N, 93°61'W, altitude 288 m),

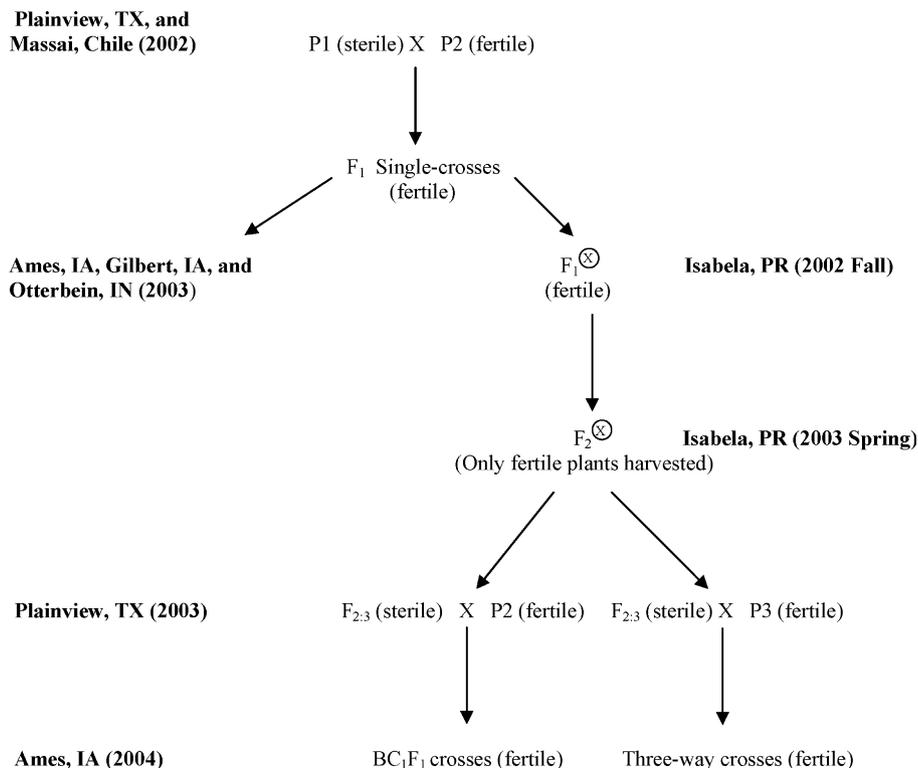


Fig. 1. Seed production scheme for single-crosses, three-way crosses, and BC_1F_1 crosses.

Gilbert, IA (42°13'N, 93°65'W, altitude 291 m), and Otterbein, IN (40°61'N, 87°32'W, altitude, 253 m) in 2003.

The crop rotation was corn, oat, and soybean. The fertilization rate was 15–40–60 (N–P–K) for corn, and 0–60–90 for oat and soybean. Soil pH was 6.6–7.3 for the experimental plots. The prevalent inoculant (*Bradyrhizobium japonicum*) serogroups 123 were applied in the neutral to acid soils, and 135 in the alkaline soils. A RCBD design with two replications was used per combination. Each replicated plot consisted of four rows, 5.1 m long, spaced 76 cm between rows, and 90 cm between plots, planted with 20 seeds per meter per row. At maturity, the two center rows of each plot were harvested to determine yield, measured as the seed weight of the two rows. Seed-protein and seed-oil content also were determined using the two center rows. Grain yield was estimated based on total weight of the two harvested rows. Seed-protein and seed-oil content (hereafter referred as protein content and oil content) were analyzed by near-infrared reflectance spectroscopy (NIR) at the National Center for Agricultural Utilization Research (NCAUR), Peoria, IL.

2.4.2. Three-way and BC₁F₁ crosses

Single-crosses were evaluated in 2003, but were not evaluated in 2002. For the 2004 tests, female and male parents of the single-crosses, three-way and BC₁F₁ crosses, plus two commercial checks, were evaluated at the Bruner Farm near Ames, IA. The crop rotation was corn, oat, and soybean. The fertilization rate was 0–0–90 (N–P–K) for oat and soybean. The same inoculants were applied following the previously described procedure. A RCBD design with two replications per combination and 16 combinations was used. At maturity, the two center rows were harvested to measure grain yield, protein and oil content. Plot size and data were recorded as described earlier.

2.5. Statistical analysis

Analyses of variance were performed for each trait using PROC GLM of SAS v. 9 (SAS Institute, 2003). For the single-cross evaluation, replications and locations were considered random effects, genotypes as fixed effects. Similar assumptions were used for the three-way and BC₁ crosses, replications were random effects, and genotypes were fixed.

Mid-parent heterosis (MPH) was calculated as:

$$\text{MPH} = \frac{F_1 - \text{MP}}{\text{MP}} \times 100$$

where F_1 is the mean of the F_1 hybrid averaged over replications and $\text{MP} = (P_1 + P_2)/2$ in which P_1 and P_2 are the means of the inbred parents for the single-crosses. For three-way and BC₁F₁ crosses, $\text{MP} = [(P_1 + P_2)/2 + P_3]/2$, in which P_1 and P_2 are the means of the inbred parents for the single-crosses and P_3 is the mean of the third parent or recurrent parent.

High-parent heterosis (HPH) was calculated as:

$$\text{HPH} = \frac{F_1 - \text{HP}}{\text{HP}} \times 100$$

where HP = mean of the best or highest parent.

Heterosis estimates, MPH and HPH were tested for being significantly different from zero by using *t*-tests. Pearson phenotypic correlation coefficients were calculated to detect associations between heterosis expression for grain yield and absolute grain yield, heterosis for grain yield and protein content, heterosis for grain yield and oil content, protein content and oil content, protein content and grain yield, and oil content and grain yield using PROC CORR of SAS v. 9 (SAS Institute, 2003).

3. Results and discussion

3.1. Single-crosses and parental performance

Significant differences in grain yield between parental lines and single-crosses were observed. The interaction effect for location × lines (parents and single-crosses) also was significant (Table 1). Average grain yield for parental lines was 2065 kg ha⁻¹ at Ames; 2185 kg ha⁻¹ at Otterbein, and 2225 kg ha⁻¹ at Gilbert. For hybrids, mean values were 1790 kg ha⁻¹ in Ames, 1797 in Gilbert, and 2381 kg ha⁻¹ in Otterbein. Crosses A00-39 *ms2* × DSR Exp. 202b and A00-41 *ms2* × DSR Exp. 202b had the highest average grain yield (2532 kg ha⁻¹ and 2512 kg ha⁻¹, respectively). The commercial cultivar DSR Exp. 202b was the parental line with the highest grain yield mean 2464 kg ha⁻¹ (data not shown).

Grain yield among single-crosses was varied, but the relative ranking for the top yielding populations was very similar across locations (data not shown). The significant genotype × environment interaction observed, may have only affected the differences in absolute yield values since the best hybrids in the best environment also performed well in the low yielding environment. Male-parent contributions to hybrid yields was noticeable for some of the genotypes, i.e., for DSR Exp. 202b and A00-68 *Ms3*. DSR Exp. 202b which presented the highest grain yield of all parental lines, produced the best yielding hybrids, although lower in yield than the male parent (DSR Exp. 202b). The same trend was observed for the lowest yielding hybrid A00-72 *ms8* (T358) × A00-68 *Ms3* (T284), which had as male parent A00-68 *Ms3* (T284), the lowest yielding parental line. These results emphasized the importance of the male parent in hybrid performance.

For protein content, no significant differences were observed for either parents or hybrids (data not shown). Mean protein values of parental lines and single-crosses were similar,

Table 1
Combined analysis of variance for grain yield, for parents, commercial checks, and single-crosses at Ames and Gilbert, Iowa, and Otterbein, Indiana (2003)

Lines	Source of variation	Mean squares
Parents and commercial checks	Lines	1,094,942**
	Location × parents	247,619**
	Error	2,207,024
Single-crosses	Lines	919,599**
	Location × hybrids	206,289**
	Error	1,153,080

** Significant at the 0.05 probability level.

although the mean value for hybrids was slightly higher than for parental lines (Table 2). The mean protein content for parental lines was 388 g kg^{-1} compared to 397 g kg^{-1} for hybrids (Table 2). Nakasathien et al. (2000) indicated that seed protein content of normal soybean cultivars can be increased by increasing the external N concentration to supra-optimal levels during reproductive growth. They suggested that developing seed have the capacity to synthesize high protein when

adequate substrate is available. Fertilization was applied to the experimental plots; it did not have an effect on seed protein content. The mean seed protein content values for parents and hybrids were average for soybean cultivars, although selection for high or low protein content was not exerted on the parents. The soil pH values measured in our experimental plots were not that low (6.6–6.9) to severely inhibit the nodulation process, so a small response could be expected from fertilization. Scharp

Table 2

Mean values for grain yield, seed protein, and seed oil content for parents, commercial checks, and single-crosses at Ames and Gilbert, Iowa, and Otterbein, Indiana (2003)

Parents and commercial checks	Grain yield (kg ha^{-1})	Protein content (g kg^{-1})	Oil content (g kg^{-1})
2003			
DSR Exp. 202b	2764	393	214
DSR 9389	2736	382	210
DSR 180	2661	393	206
DSR 218	2527	394	220
Corsoy 79	2298	393	210
A94-20 \times 19 (Ms6)	2117	403	189
A00-72 Ms8 (T358)	2085	379	182
A00-63 Ms2 (Beeson)	2000	378	201
Hark	1988	396	206
A00-61 Wells	1894	390	195
A00-41 Ms2 (ARS-10-483)	1776	386	216
A00-39 Ms2 (ARS-10-483)	1991	376	202
A00-73 Ms9 (T359)	1649	386	200
A00-68 Ms3 (T284)	1590	381	179
Mean	2156	388	202
LSD ($\alpha = 0.05$)	167	24	13
C.V.	17.83	2.59	2.58
Single-crosses			
Mean	1993	397	196
LSD ($\alpha = 0.05$)	334.74	22.10	18.48
C.V.	11.13	2.30	3.88
2004			
DSR Exp. 2002b	3038	403	191
GH 4190	2727	396	188
GH 4189	2666	394	185
Raiden	2535	413	173
Hark	2503	419	184
A00-41 Ms2 (ARS-10-483)	2368	403	181
Corsoy 79	2275	398	184
A00-39 Ms2 (ARS-10-483)	2263	416	182
A94-20 \times 19 (Ms6)	2222	399	172
A00-63 Ms2 (Beeson)	2222	378	182
Wells	2199	405	181
A00-72 Ms8 (T358)	2000	389	185
A00-73 Ms9 (T359)	2146	404	179
A00-68 Ms3 (T284)	1924	414	177
Mean	2406	404	182
LSD ($\alpha = 0.05$)	789.91	23.07	9.24
C.V.	13.88	2.41	2.14
Three-way crosses			
Mean	2648	408	184
LSD ($\alpha = 0.05$)	583.74	22.57	6.28
C.V.	9.18	2.39	1.48
BC₁F₁ crosses			
Mean	2659	411	181
LSD ($\alpha = 0.05$)	785.03	12.03	11.16
C.V.	12.80	1.26	2.66

and Wiebold (2003) reported 62.25 kg/Ha response to N applied at planting for sites with soil pH less than 6.0, and for sites with soil nitrate less than 56.05 kg/Ha in the top 60 cm.

Oil content was not significantly different among single-crosses, although it was among parental lines (data not shown). Contrary to protein content, mean oil content among parental lines was slightly higher when compared to hybrids (Table 2). The mean oil content for parental lines was 202 g kg⁻¹ compared to 196 g kg⁻¹ for hybrids (Table 2).

3.1.1. Heterosis

Across locations, positive and negative heterosis values for grain yield were observed for single-crosses. MPH values for grain yield at each location ranged from -59 to +37%, whereas the range for HPH was from -65% to +16% (data not shown). The highest average MPH value across locations was for cross A00-41 *ms2* × A00-73 *Ms9* (T359), while hybrid A00-72 *ms8* (T358) × A00-68 *Ms3* (T284) had the lowest average MPH and HPH (Table 3).

For protein content, MPH values ranged from -0.62% to +5% and HPH from -4% to +3%. The hybrid A00-39 *ms2* × DSR Exp. 202b showed the highest protein value for

both MPH and HPH, and the hybrid [A94-20 × 19 (*ms6*)] × A00-39 *Ms2*, the lowest value for both heterosis estimates (Table 4).

The MPH and HPH estimates for grain yield observed in our study for single-crosses were in the range that has been reported in the literature, where high-parent heterosis values from -39% (Weber et al., 1970), +8% (Paschal and Wilcox, 1975), +26% (Chauhan and Singh, 1982), and +48% (Tain, 1981) have been reported. More recently, Lewers (1996), reported F₁ MPH for grain yield ranging from -0.43 to +15.61%, Manjarrez-Sandoval et al. (1997) reported MPH for grain yield ranging from +0.8 to +15% for F₂ plants.

In our study, superiority of some hybrids for yield over their mid-parent value for yield suggested that gene action other than additive may have been present. The cross-combinations evaluated were chosen without any previous knowledge about combining ability, but rather on traits of the parents *per se* related to pollinator attraction in one parent (mainly the female parent, but also in some of the male parents), and agronomic traits in the other. Little data about parental evaluation to determine combining ability among soybean lines was available, which complicated parental selection. The narrow

Table 3
Mid-parent value (MPV), mid-parent heterosis (MPH), high-parent heterosis (HPH), and average heterosis for grain yield for single-crosses at Ames and Gilbert, Iowa, and Otterbein, Indiana (2003), three-way crosses at Ames, Iowa (2004), and BC₁F₁ crosses at Ames, Iowa (2004)

	MPV (kg ha ⁻¹)	MPH (%)	HPH (%)
2003			
Single-crosses			
A00-39 <i>ms2</i> × DSR Exp. 202b	2378	+4.77	-8.62
A00-41 <i>ms2</i> × DSR Exp. 202b	2270	+9.71	-9.15
A00-39 <i>ms2</i> × Corsoy 79	2145	+7.62	-5.49
A94-20 × 19 (<i>ms6</i>) × A00-39 <i>Ms2</i>	2054	+6.11	+2.92
A00-41 <i>ms2</i> × A00-73 <i>Ms9</i> (T359)	1713	+15.30	+11.19
A00-68 <i>ms3</i> (T284) × A00-41 <i>Ms2</i>	1683	+5.89	-3.53
A00-63 <i>ms2</i> (Beeson) × Wells	1947	-8.96	-18.98
A00-39 <i>ms2</i> × Hark	1990	-13.47	-23.11
A00-72 <i>ms8</i> (T358) × A00-68 <i>Ms3</i> (T284)	1837	-34.30	-41.11
Average heterosis		-0.81	-10.65
2004			
Three-way crosses			
(A00-39 <i>ms2</i> × Corsoy 79) × DSR Exp. 202b	2654	-5.67	-17.61
[A94-20 × 19 (<i>ms6</i>) × A00-39 <i>Ms2</i>] × DSR Exp. 202b	2640	-13.95	-25.20
(A00-41 <i>ms2</i> × A00-73 <i>Ms9</i>) × DSR Exp. 202b	2648	+7.01	-6.73
(A00-68 <i>ms3</i> × A00-41 <i>Ms2</i>) × DSR Exp. 202b	2592	+4.79	-10.58
[A00-63 <i>ms2</i> (Beeson) × Wells] × DSR Exp. 202b	2624	-1.83	-15.20
(A00-39 <i>ms2</i> × Hark) × DSR Exp. 202b	2711	+5.71	-5.67
(A00-72 <i>ms8</i> × A00-68 <i>Ms3</i>) × DSR Exp. 202b	2500	+16.04	-4.50
(A00-73 <i>ms9</i> × Raiden) × DSR Exp. 202b	2689	+1.01	-10.58
Average heterosis		+1.63	-12.00
BC ₁ F ₁ crosses			
(A00-39 <i>ms2</i> × Corsoy 79) × Corsoy 79	2272	+17.63	+17.48
[A94-20 × 19 (<i>ms6</i>) × A00-39 <i>Ms2</i>] × A00-39 <i>Ms2</i>	2253	+22.25	+21.70
(A00-41 <i>ms2</i> × A00-73 <i>Ms9</i>) × A00-73 <i>Ms9</i>	2202	+16.20	+8.02
(A00-68 <i>ms3</i> × A00-41 <i>Ms2</i>) × A00-41 <i>Ms2</i>	2257	-11.39	-15.55
[A00-63 <i>ms2</i> (Beeson) × Wells] × Wells	2216	+42.34	+41.97
(A00-39 <i>ms2</i> × Hark) × Hark	2443	+5.92	+3.36
(A00-72 <i>ms8</i> × A00-68 <i>Ms3</i>) × A00-68 <i>Ms3</i>	1962	+40.68	+38.00
(A00-73 <i>ms9</i> × Raiden) × Raiden	2437	+14.32	+9.94
Average heterosis		+18.49	+17.05

Table 4

Mid-parent value (MPV), mid-parent heterosis (MPH), high-parent heterosis (HPH), and average heterosis for seed protein content and seed oil content for single-crosses at Ames and Gilbert, Iowa, and Otterbein, Indiana (2003), three-way crosses at Ames, Iowa (2004), and BC₁F₁ crosses at Ames, Iowa (2004)

	Protein content			Oil content		
	MPV (g kg ⁻¹)	MPH (%)	HPH (%)	MPV (g kg ⁻¹)	MPH (%)	HPH (%)
2003						
Single-crosses						
A00-39 <i>ms2</i> × DSR Exp. 202b	385	+5.81	+3.53	208.25	-2.11	-3.98
A00-41 <i>ms2</i> × DSR Exp. 202b	390	+2.12	+1.19	215.40	-5.61	-6.01
A00-39 <i>ms2</i> × Corsoy 79	384	+1.87	-0.22	206.25	-0.87	-2.89
A94-20 × 19 (<i>ms6</i>) × A00-39 <i>Ms2</i>	390	-1.08	-4.34	195.35	-1.89	-5.14
A00-41 <i>ms2</i> × A00-73 <i>Ms9</i> (T359)	386	+2.83	+2.82	208.15	-9.39	-12.76
A00-68 <i>ms3</i> (T284) × A00-41 <i>Ms2</i>	383	+2.35	+1.63	197.75	-5.05	-13.22
A00-63 <i>ms2</i> (Beeson) × Wells	384	+3.88	+2.33	197.90	+0.70	-0.84
A00-39 <i>ms2</i> × Hark	386	-0.62	-3.12	204.15	-1.71	-2.76
A00-72 <i>ms8</i> (T358) × A00-68 <i>Ms3</i> (T284)	380	+5.30	+2.90	192.25	-6.71	-12.66
Average heterosis		+2.49	+0.74		-3.62	-6.69
2004						
Three-way crosses						
(A00-39 <i>ms2</i> × Corsoy 79) × DSR Exp. 202b	406	+0.46	-2.72	186.77	-2.51	-4.50
[A94-20 × 19 (<i>ms6</i>) × A00-39 <i>Ms2</i>] × DSR Exp. 202b	405	-0.14	-1.17	183.75	-2.34	-5.87
(A00-41 <i>ms2</i> × A00-73 <i>Ms9</i>) × DSR Exp. 202b	402	+0.14	-0.09	185.47	+1.56	-1.20
(A00-68 <i>ms3</i> × A00-41 <i>Ms2</i>) × DSR Exp. 202b	406	-0.78	-0.43	184.83	-0.97	-3.98
[A00-63 <i>ms2</i> (Beeson) × Wells] × DSR Exp. 202b	397	+3.80	+1.92	186.13	-1.77	-4.09
(A00-39 <i>ms2</i> × Hark) × DSR Exp. 202b	410	-0.20	-1.65	186.80	+0.53	-1.46
(A00-72 <i>ms8</i> × A00-68 <i>Ms3</i>) × DSR Exp. 202b	407	+2.23	-0.55	184.97	-2.05	-4.98
(A00-73 <i>ms9</i> × Raiden) × DSR Exp. 202b	405	-0.39	-2.49	183.50	+1.58	-2.20
Average heterosis		+0.64	-0.90		-0.30	-2.87
BC ₁ F ₁ crosses						
(A00-39 <i>ms2</i> × Corsoy 79) × Corsoy 79	402	+0.77	-2.57	183.52	+1.08	+0.76
[A94-20 × 19 (<i>ms6</i>) × A00-39 <i>Ms2</i>] × A00-39 <i>Ms2</i>	412	-0.92	-1.94	179.35	-1.11	-2.47
(A00-41 <i>ms2</i> × A00-73 <i>Ms9</i>) × A00-73 <i>Ms9</i>	401	+0.09	-0.44	179.99	+0.27	-0.44
(A00-68 <i>ms3</i> × A00-41 <i>Ms2</i>) × A00-41 <i>Ms2</i>	406	+0.88	-1.04	180.12	-0.55	-1.05
[A00-63 <i>ms2</i> (Beeson) × Wells] × Wells	398	+2.71	+1.03	181.37	+0.82	+0.32
(A00-39 <i>ms2</i> × Hark) × Hark	418	-0.86	-1.02	183.60	-0.98	-1.30
(A00-72 <i>ms8</i> × A00-68 <i>Ms3</i>) × A00-68 <i>Ms3</i>	412	+1.79	+1.47	178.12	+1.74	-0.33
(A00-73 <i>ms9</i> × Raiden) × Raiden	410	+2.51	+1.69	174.85	+4.97	+2.22
Average heterosis		+0.87	-0.35		+0.78	-0.28

germplasm base in soybean in US maturity groups might complicate the selection of parents of different origin as well. Gizlice et al. (1994) reported that only six ancestors constituted more than half of the genetic base of North American soybean germplasm. In spite of this complication, when crosses were grouped by families sharing common female parental lines, the MPH estimates for each family showed a pattern of combining ability among some parental lines. For example, the MPH mean value for the families with A00-39 *ms2* (ARS-10-483) as female parent was +3.21%, lower when compared to the families that included its sister line A00-41 *ms2* (ARS-10-483) as female parent MPH (+12.05%) (Fig. 2). For crosses that included A00-39 *ms2* as female parent, negative MPH estimates were observed in single- and three-way crosses. Conversely, for A00-41 *ms2* as female parent, positive estimates were observed across all its combinations.

In spite of these observations, overall grain yield of single-crosses was lower when compared to checks and the parental lines. With the HPH estimates mainly negative, a possible explanation for these results could be the large difference in

yield among parental lines of each cross. The common male parent, line DSR Exp. 202b is a commercial line with high yield. The female parents used in the present study were mainly genetic stocks, while the recurrent male parents were either the

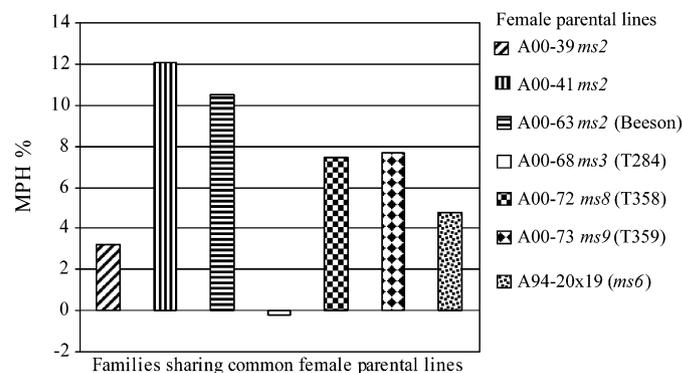


Fig. 2. Mid-parent heterosis (MPH) mean values for grain yield for single-crosses, three-way crosses, and BC₁F₁ crosses when grouped by families (sharing common female parental lines).

Table 5

Pearson phenotypic correlation coefficients among heterosis for grain yield, seed protein content, and seed oil content for single-crosses at Ames and Gilbert, Iowa, and Otterbein, Indiana (2003), three-way crosses at Ames, Iowa (2004), and BC₁F₁ crosses at Ames, Iowa (2004)

Traits	Protein content	Oil content	Grain yield
Single-crosses			
Heterosis for grain yield	0.93**	0.04NS	0.74***
Seed protein content		−0.05NS	0.53NS
Seed oil content			0.17NS
Three-way crosses			
Heterosis for grain yield	0.04NS	−0.29NS	−0.25NS
Seed protein content		0.60NS	−0.38NS
Seed oil content			0.65*
BC ₁ F ₁ crosses			
Heterosis for grain yield	0.35NS	0.11NS	0.65*
Seed protein content		0.15NS	0.14NS
Seed oil content			0.37NS

NS = not significant.

* Significant at the 0.05 level.

** Significant at the 0.01 level.

*** Significant at the 0.001 level.

fertile siblings of the female parents, plant introductions, or cultivars released more than 20 years ago. Thus, large differences in yield were expected. Within the female parent group, large differences also were observed. Some of the female parents showed potential for yield for the area where the experiments were conducted. Others presented a very poor performance (Table 2), even though some of those lines were grown in their geographical area of adaptation. These results emphasize the importance of parent selection to predict hybrid performance.

For single-crosses, the only significant phenotypic correlations were between yield heterosis with protein content and grain yield (Table 5). Pair-wise correlations of grain yield with protein content, grain yield with oil content, and protein content with oil content were not significant (Table 5).

3.2. Three-way crosses, BC₁F₁ crosses, and parental performance

There were no significant differences for grain yield or protein content among three-way crosses, or their parental lines. Oil content was significantly different only for parental lines. The commercial parental line DSR Exp. 202b had the highest mean oil content; the lowest was observed in A94-20 × 19 (*Ms6*) (Table 3).

There were no significant differences for grain yield for parental lines of the BC₁F₁ crosses, only among BC₁F₁ crosses. The highest yielding hybrid was the cross [A00-63 *Ms2* (Beeson) × Wells] × Wells (3155 kg ha^{−1}), the lowest value was for the cross (A00-68 *ms3* × A00-41 *Ms2*) × A00-41 *Ms2* (2000 kg ha^{−1}).

Significant differences for protein content were not observed for parental lines, only among BC₁F₁ hybrids. The hybrid with the highest protein mean was cross (A00-73 *ms9* × Rai-Raiden) × Raiden. Absence of nitrogen application was

observed to have a differential impact only on the seed protein content in the hybrids. Nitrogen fertilization was not recommended based upon tests conducted by The Iowa State University Soil and Plant Analyses Laboratory. N₂ fixation was not measured; plants were heavily nodulated, however, we could not assess the effect of those processes in the final protein content accumulated in the plants. Oil content was not significantly different for BC₁F₁ crosses or their parental lines.

3.2.1. Heterosis

For the three-way crosses, positive and negative MPH values were observed for grain yield. The HPH values in contrast were all negative. The three-way cross (A00-72 *ms8* × A00-68 *Ms3*) × DSR Exp. 202b had the highest MPH value, the lowest MPH value was for cross [A94-20 × 19 (*ms6*) × A00-39 *Ms2*] × DSR Exp. 202b (Table 3). For protein content, heterosis values had a narrower range than for grain yield for both estimates, ranging from −0.78 to +3.80 for MPH, and from −2.72 to +1.92 for HPH. The three-way cross [A00-63 *Ms2* (Beeson) × Wells] × DSR Exp. 202b had the highest MPH and HPH values, the three-way cross (A00-39 *ms2* × Corsoy 79) × DSR Exp. 202b showed the lowest HPH value (Table 4). Considering all three-way combinations only 14% of the three-way crosses had positive heterosis values, compared to 28% of the BC₁F₁ crosses. A similar trend also was observed for oil content, i.e., heterosis estimates were small in value and had a narrow range (Table 4). The three-way cross (A00-73 *ms9* × Raiden) × DSR Exp. 202b had the highest MPH value, the three-way cross [A94-20 × 19 (*ms6*) × A00-39 *Ms2*] × DSR Exp. 202b presented the lowest HPH value (Table 4). For the three-way crosses, heterosis for grain yield was significantly correlated only with oil content (Table 5).

Positive and negative heterosis for grain yield also was observed for BC₁F₁ crosses. The BC₁F₁ cross [A00-63 *ms2* (Beeson) × Wells] × Wells had the highest HPH, the lowest was for cross (A00-68 *ms3* × A00-41 *Ms2*) × A00-41 *Ms2* (Table 3). BC₁F₁ crosses as a group showed the highest MPH and HPH values when compared to single-crosses, and three-way crosses (Table 3).

For protein content, heterosis values ranged from −0.92% to +2.71% for MPH, and from −2.57% to +1.69% for HPH. The BC₁F₁ cross [A00-63 *ms2* (Beeson) × Wells] × Wells had the highest MPH value. The BC₁F₁ cross (A00-39 *ms2* × Corsoy 79) × Corsoy 79 presented the lowest HPH value (Table 4). For oil content, BC₁F₁ cross (A00-73 *ms9* × Raiden) × Raiden had the highest value. The BC₁F₁ cross [A94-20 × 19 (*ms6*) × A00-39 *Ms2*] × A00-39 *Ms2* presented the lowest MPH and HPH values (Table 4). For BC₁F₁ crosses, heterosis for grain yield was significantly correlated only with grain yield.

The three-way cross [A94-20 × 19 (*ms6*) × A00-39 *Ms2*] × DSR Exp. 202b, one of the crosses with the lowest mean for grain yield, also showed the lowest HPH for grain yield, the lowest mean for oil content, and the lowest HPH for oil content. The highest yielding hybrid BC₁F₁ [A00-63 *ms2* (Beeson) × Wells] × Wells, conversely, presented the highest

HPH for grain yield, and had both positive heterosis and mean values for protein and oil content, which also was observed in BC₁ crosses (A00-72 *ms8* × A00-68 *Ms3*) × A00-68 *Ms3*, and (A00-73 *ms9* × Raiden) × Raiden. These results suggest that in these crosses, alleles with the expected pleiotropic effect of high grain yield and low protein content, or high oil content with low protein content reported for pure line cultivars, were not present. Estimates of genetic correlations between yield and protein content in breeding populations are usually negative (Burton, 1985, 1987). Positive correlations for traits of interest are preferred. However, the fact that non-significant correlations were observed was of interest. Such non-significant correlations included heterosis for grain yield with protein content, grain yield with protein content, and protein content with oil content among three-way crosses, and BC₁F₁ crosses. The results in the present study are not in agreement with the observations by Wilcox and Cavins (1995), who reported a moderate to strong inverse relationship between seed yield and protein content ranging from $r = -0.23$ to -0.86 in successive backcross cycles to transfer high seed protein to a recurrent parent cultivar. Burton (1987) also reported this inverse relationship. Because of the negative correlation between these traits, methods to select and improve them simultaneously typically require large populations derived from many different parental combinations.

3.3. Heterosis across populations

When average MPH and HPH values for grain yield from single-crosses, three-way crosses, and BC₁F₁ crosses were compared, it was observed that going from single-crosses to three-way crosses resulted in a decrease in both MPH and HPH in four of the eight crosses. This is in contrast to what was observed going from single-crosses to BC₁F₁ crosses, where seven of the eight crosses had an increase in both MPH and HPH. Single-crosses, three-way crosses, and BC₁F₁ crosses, which shared common parental lines, were grouped in families and compared for MPH mean values for grain yield (Fig. 2). The highest MPH mean was observed for the family comprised of crosses that had A00-41 *ms2* as female parent. The lowest MPH mean was observed for the family that comprised crosses that had A00-68 *ms3* as female parent.

Additive gene action has been reported as a main component in the genetic variance for soybean (Brim and Cockerham, 1961). Models to explain heterosis in self-pollinated crops based on additive × additive epistasis have been proposed (Compton, 1977), and confirmed in peanut (Isleib et al., 1978), and oat (Pixley and Frey, 1991). The fact that BC₁F₁ crosses showed heterosis might suggest that additive × additive epistasis, which does not contribute to inbreeding depression (Compton, 1977; Lamkey and Edwards, 1999), could be present. The heterosis estimates for grain yield of the BC₁F₁ crosses were higher compared to their single-cross and three-way cross counterparts when evaluated in a common environment (Ames). However, trying to make some comparisons among these hybrids could be very problematic and only can suggest some patterns in the

performance of these hybrids presenting different genetic makeup. Because only single-crosses were replicated on several locations, it was not possible to remove the G × E interaction effect from the genetic variance in the BC₁F₁ and three-way crosses. The single-crosses should be replicated in more locations to provide meaningful results. Thus, the results observed might only suggest trends that need to be confirmed in replicated environments. Also, the feasibility of using BC₁ populations to produce hybrids must be addressed considering the heterosis levels that might be obtained. However, the fact that more seasons are needed to produce the hybrid F₁ seed compared to single-crosses may present some disadvantages.

4. Summary

A limiting factor for developing efficient hybrid seed production in soybean has been pollen transfer from the male parent to the female parent. In our study, we used insect pollinators to produce large quantities of hybrid seed to conduct replicated agronomic tests in multiple locations. Agronomic and yield data suggested that heterosis is possible in some of the crosses tested. Heterosis is a dynamic attribute strongly affected by the environment; nonetheless, the results revealed that hybrid vigor was present for seed yield. Additionally, these hybrids did not have significant differences in protein and oil content when compared to the best parent. Positive heterosis levels were observed for some traits across all populations tested, although these might indicate trends that have to be considered carefully. For more conclusive results it is imperative that testing of more cross-combinations be done in replicated environments. Extensive research in different environments must be conducted to determine what parental combinations will produce the highest heterosis levels, and to develop criteria for selecting the parents with the best combining ability. This will be important to maximize heterosis expression that can economically justify the use of hybrids in soybean production.

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