UDC 575.630
https://doi.org/10.2298/GENSR2201265K
Original scientific article

ASSESSMENT OF EFFICIENCY OF BREEDING METHODS USING MOLECULAR MARKERS IN SOYBEAN

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Khosla G., B S Gill, A. Sirari, P. Singh (2022). *Assessment of efficiency of breeding methods using molecular markers in soybean*. - Genetika, Vol 54, No.1, 265-274.

Four breeding methods *viz.* pedigree method (PM), single pod descent (SPD), single pod descent with selection (SPDS) and bulk method (BM) were compared for maintaining variability in the population in advanced generations using simple sequence repeats (SSR) markers. The $F_{4:7}$ lines advanced through different breeding methods from six different crosses were evaluated for number of unique lines retained in each method at a similarity coefficient $\geq$ 0.875. Eighteen polymorphic SSR markers were used for estimating similarity coefficient between lines within a breeding method in each cross. In all the crosses, SPD method was the best method in producing unique lines with a range from 42.9 to 100 per cent. SPD method had also the least number of lines pairing with two or more lines. PM and BM had the least number of unique lines in three crosses each and also maximum proportion of lines produced by these two methods were paired with four or more lines. Thus, SPD method was the most efficient among these four methods in retaining the variability in a population, but the breeder has to make a choice between high variability and comparative harvest and seed processing efficiencies to select the most suitable breeding method.

Keywords: Soybean, Breeding methods, Similarity coefficient, SSR markers

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is a self-pollinated species with less than one per cent out-crossing (SHURTLEFF and AOYAGI, 2007) belonging to Leguminosae family and the subfamily Papilionideae. Soybean is a suitable option for increased productivity because of wider geographical adaptation and diverse outputs (HAYATI *et al.*, 2009; MOSER, 2011). It generates both protein and oil; the proteins contain essential amino acids vital for vegetarian people (RACKIS *et al.*, 1961); whereas its oil is having some distinct properties to make it ideal for edible and industrial uses (HAYATI *et al.*, 2009; HOSSAIN and AL-SAIF, 2010).

Breeders attempt several types of crosses between varieties or germplasm lines to alter gene frequency in the breeding population via gene recombination. Handling a mating scheme and a breeding population is crucial in providing increased potential for genetic superiority (SCABOO *et al.*, 2010). For this, breeders choose methods which help in simultaneous improvement of yield and component traits. In soybean, traditional breeding methods involving

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hybridization and phenotypic selection are responsible for all the genetic gain in yield (CORYELL *et al.,* 1999). Many breeding methods of generation advance *viz.* bulk, pedigree, single seed descent, early generation testing and their modifications have been proposed and used for soybean improvement. Efficiency of these methods has been compared based on generation of superior lines (KHOSLA *et al.,* 2019). However, conclusions drawn based on field studies are contradictory. Extent of genetic variation present in the lines isolated from a particular breeding method can be used to assess the efficiency of breeding method because genetic variability is the key factor determining the extent to which a population can be improved. Breeders look for maximizing the genetic variance initially by selection of diverse parents and then by appropriate breeding method for generation advance. Appropriate breeding method is one that avoids repetitive sampling of a single genotype in segregating generations. Repetitive sampling results in accumulation of redundant lines in the population. In an advance generation like F_5 or F_6, genetic variation is reduced if multiple lines are contributed by a single F_2 plant. Assessment of genetic variability through molecular markers retained in the populations advanced by different methods can be used to compare the efficiency of breeding methods. The single seed descent method has been compared to single pod descent and bulk method using computer simulation studies (MUEHLBAUER *et al.,* 1981; KERVELLA and FOUILOUX, 1992). However, only single citation is available in literature in which single pod descent, single seed descent and bulk method has been compared using SSR markers (FUNADA *et al.,* 2013). No study is available in which efficiency of pedigree, bulk, single pod descent and single pod descent with selection methods has been compared using molecular markers. Therefore, our objective was to determine the relative efficiency of the commonly used methods of generation advance *viz.*, pedigree (PM), single pod descent (SPD), single pod descent with selection (SPDS) and bulk method (BM) using SSR markers in soybean. The relative efficiency of each of the four inbreeding methods was defined as the number of inbred lines that were not paired with any other line, at a specified coefficient of similarity level.

MATERIALS AND METHODS

Development of material

The present study was carried out at Punjab Agricultural University, Ludhiana. Six soybean crosses viz. [(SL 525 x AGS 328) x {(SL 744 x SL 682) x AGS 752}], SL 525 x {(AGS 328 x SL 682) x AGS 752}, SL 744 x (SL 525 x AGS 328), SL 755 x SL 794, SL 525 x IC 391477, SL 525 x Lsb 23 were attempted during *Kharif,* 2008. Sufficient pollinations were attempted for each cross combination and seeds were set in 4-8 pods in various crosses. About 11-15 F_1 seeds were space planted in different crosses during the *Kharif,* 2009. Seeds were harvested from each F_1 plant in each cross separately. The F_2 populations of these crosses ranging from 645 to 913 plants were space planted during *Kharif,* 2010 which was further advanced through four different methods of generation advancement viz. pedigree method, single pod descent with selection, single pod descent and bulk method. For pedigree selection about 50 plants were visually selected in F_2 generations. In single pod descent with selection method, single pods with three seeds from the selected F_2 plants were harvested and seeds from these pods were bulked for each cross to grow F_3 generation. In single pod descent method, single pods with three seeds were taken from each F_2 plant, including the plants selected for

pedigree method and SPDS method and bulked to grow the F_3 generation. In the bulk method, all the plants in F_2 were harvested in bulk and a sample (150 gram) from this was used to grow 15 rows per cross till F_4 generation. Seeds from plants selected for pedigree method, single pod descent method and single pod descent with selection method were also added to the bulk in F_2.

In F_5 generation, single plant selections were made from all the four methods and plants to progeny rows were grown in F_6 and F_7 generations during *Kharif* 2014 and *Kharif* 2015, respectively. All segregating progenies were discarded and seed of uniform progenies was multiplied for further evaluation. During *Kharif* 2016, the progenies obtained from all the six crosses were used to compare the efficiency of breeding methods.

DNA extraction and SSR marker analysis

Young leaves were collected from a single plant in each line. Genomic DNA of these plants was isolated using the CTAB (Cetyltrimethyl ammonium bromide) method as given by SAGHAI-MAROOF *et al.,* (1984). Extracted DNA pallets were dissolved in 1X TE (Tris EDTA buffer–10mM TrisHCl, 1mM EDTA, pH 8.0) buffer and the dissolved DNA was stored at -20°C for further use. The quantity and quality of DNA were assessed by electrophoresis in 0.8% agarose gels with known concentrations of DNA marker. The quality of DNA samples was judged based on whether DNA formed a single band of high molecular weight (good quality) or a smear (degraded/poor quality). The lines were evaluated with 18 SSR markers (Table 1). SSR primers were synthesized by IDT Company (India).

Table 1. SSR markers used for genotyping soybean lines

Sr. No.	Marker name	Linkage group	Position (cM)	Sr. No.	Marker name	Linkage group	Position (cM)
1	Satt619	A1	69.21	10	Sat_183	D1b	112.63
2	Sct_067	A2	14.99	11	Satt543	D2	88.02
3	BE806308	B1	0.00	12	Satt301	D2	93.71
4	Satt426	B1	28.33	13	Satt554	F	111.89
5	Satt197	B1	46.39	14	AW756935	F	124.88
6	Satt560	B2	97.92	15	Satt353	H	8.48
7	Satt371	C2	145.48	16	Satt354	I	46.22
8	Satt184	D1a	17.52	17	Satt588	K	117.02
9	Satt129	D1a	109.67	18	Satt153	O	118.14

In vitro DNA amplification through PCR was performed in a 96 well microtiter plate in a Veriti thermal cycler™. The total reaction mixture of 20µl contained 20 ng template DNA, 100 mM of dNTPs, 1X PCR green reaction buffer, 1.5 mM $MgCl_2$, 0.25 mM of each forward and reverse primers and 1.0 unit of *Taq* DNA polymerase. The following thermal profile was used for PCR amplification: initial denaturation at 94°C (4 minutes) followed by 35 cycles of denaturation at 94°C (1 minute), annealing at 45–62°C (vary with the primers) for 1 minute and extension at 72°C (1 minute) followed by final extension at 72°C (7 minutes). A negative control (without DNA) was also used in each plate in each reaction. The PCR products were resolved on

2.5 per cent agarose gel with 100bp ladder for allele sizing and visualized under UV transilluminator and photographed using Alpha Imager EP gel documentation system.

Statistical analysis

MCCLEAN *et al.* (2012) suggested that 18 markers were adequate to determine genetic similarity in *Phaseolus vulgaris* L. Thus, in present study, 18 polymorphic primers were used to compare efficiency of four breeding methods in six crosses. Genotypes that were homozygous showed only one molecular marker band on the agarose gel and were given a score of either 0 for the M_1M_1 genotype or a score of 2 for the M_2M_2 genotype (Table 2). Heterozygous genotypes showed two bands on the agarose gel and a score of 1 was allotted for that molecular marker. If two different lines, within the same sampling method, were both homozygous for the same band at the same SSR locus, then N_{xy} equals 2 for that locus (Table 2). N_{xy} is defined as the summation of the number of alleles that two genotypes have in common across all molecular marker loci. If two lines were both homozygous, but had no SSR bands in common, then $N_{xy} = 0$ for that locus. For example if one genotype had a marker genotype of M_1M_1 and another genotype had a marker genotype of M_2M_2, at the same SSR locus, then these two genotypes had different banding patterns on the agarose gel and $N_{xy}= 0$, for that locus. If one genotype was homozygous and the other genotype was heterozygous at the same SSR locus, then these two genotypes had one allele in common, so $N_{xy} = 1$ for that marker locus. If both lines were heterozygous at the same marker locus, then $N_{xy} = 2$ for that locus because the lines have two alleles in common.

Table 2. Theoretical combinations of genotypes at a single SSR locus for two different genotypes

First genotype	Second genotype	Score of first genotype	Score of second genotype	N_{xy}
M_1M_1	M_1M_1	0	0	2
M_1M_1	M_1M_2	0	1	1
M_1M_1	M_2M_2	0	2	0
M_1M_2	M_1M_2	1	1	2
M_1M_2	M_2M_2	1	2	1
M_2M_2	M_2M_2	2	2	2

The coefficient of similarity [$S_{xy}= 2\Sigma N_{xy}/ (\Sigma N_x+ \Sigma N_y)$] was calculated as described by KEIM *et al.* (1994). Where ΣN_x is two times the total number of molecular markers that were scored for genotype X, summed across all molecular markers; and ΣN_y is two times the total number of molecular markers that were scored for genotype Y, summed across all molecular markers. The numerator of S_{xy} was equal to two times the sum of the N_{xy}.

To estimate the efficiency of different breeding methods, gel images for all the markers were transformed into binary matrix. The data on the gel produced by SSR markers were scored manually by assigning '0' for the absence of band and '1' for the presence of band for each locus. Software DARwin6 was used to study the dissimilarity coefficient between lines derived using single selection method in a cross. Dissimilarity matrices were constructed by following Unweighted Pair Group Method with Arithmetic Mean (UPGMA) function.

Efficiencies of different breeding methods were compared based on the number of unique lines retained in each breeding method. Two proportion Z-test was conducted to ascertain whether two breeding methods pooled over crosses differ significantly in terms of generating unique lines. According to KEIM *et al.* (1994) two F_4 derived lines from the same F_2 parent plant would be expected to have 75 per cent identical alleles. Similarly, two F_4 derived lines from the same F_3 parent plant would be expected to have 87.5 per cent of their alleles identical by descent. Because $F_{4:7}$ lines were evaluated in this experiment, we used the similarity coefficient (S_{xy}) of more than 0.875 to classify paired lines. Unique lines were defined as lines that were not paired with any other line at the $S_{xy} \geq 0.875$ level.

RESULTS

The efficiency of the breeding methods was calculated based on the number of unique lines developed by each method. For this purpose, dissimilarity coefficients were calculated using 18 polymorphic SSR markers (Figure1) for each pair of lines within each breeding method in all the six crosses and results are presented in Table 4.

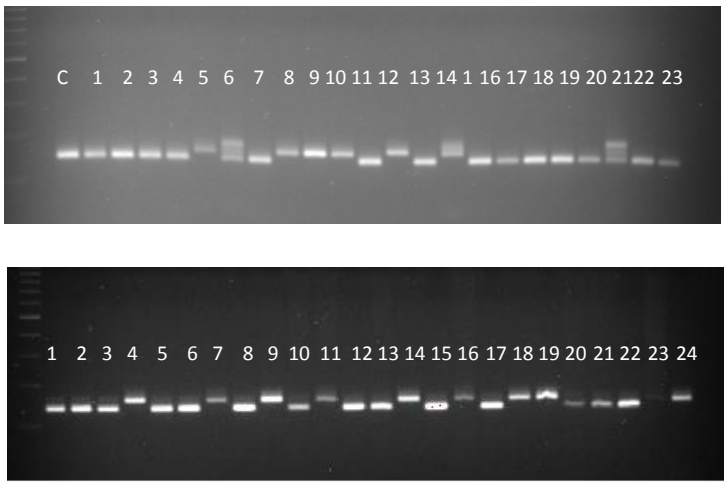

Figure 1. The amplification profiling of soybean lines with different SSR markers (Satt197 and Sct_067)

On the basis of dissimilarity coefficients, number of unique lines in each breeding method was calculated. Two proportion z-test applied on pooled data from different crosses for comparison of number of unique lines produced using different breeding methods revealed that SPD method was significantly better than the other three methods viz. PM, SPDS and BM, whereas differences were non-significant among PM, SPDS and BM (Table 3). Comparisons were also made among various breeding methods in individual crosses. In cross 1, SPD method produced 100 per cent unique lines. Bulk method was the second best method with 26.7 per cent unique lines (Table 4). 33 per cent lines in BM were paired with 2 other lines, while 20 per cent lines were paired with four or more lines. In PM, only 16.7 per cent unique lines were there and 66.7 per cent lines were paired with four or more lines. In cross 2, again SPD method had the

highest proportion of unique lines (66.7%) while remaining 33.3 per cent lines were paired with only one other line (Table 4). SPDS was the second best method with 50 per cent unique lines and the remaining lines were either paired with one line (33.3%) or two lines (16.7%). Bulk method had 33.3 per cent unique lines, while PM had only 9.1 per cent unique lines. Moreover, 36.4 per cent lines in PM were paired with four or more lines.

Table 3. Comparison of breeding methods (pooled over crosses) for producing unique lines

Breeding Method	Unique lines (%)	Z values		
SPD	68.4	$Z_{SPD/\ SPDS} = 2.70$*	$Z_{SPD/PM} = 4.01$*	$Z_{SPD/BM} = 4.47$*
SPDS	36.4	$Z_{SPDS/PM} = 0.78$	$Z_{SPDS/BM} = 1.06$	
PM	28.8	$Z_{PM/BM} = 0.31$		
BM	26.6			

*Differ significantly at 5% level of significance

In cross 3, hundred per cent lines developed through SPD method were unique. SPDS and BM were at par with 57.1 and 53.8 per cent unique lines, respectively (Table 4). In PM, only 27.3 per cent unique lines were generated. In this cross 54.5 per cent lines in PM were paired with 3 lines each. In cross 4, SPD method with 50 per cent unique lines was again the best method. BM was the second best method with 25 per cent unique lines but a high proportion of lines (60%) developed through BM were paired with four or more lines (Table 4). PM had 21.4 per cent unique lines whereas SPDS method had 20 per cent unique lines. In cross 5, SPD and SPDS methods were at par with 42.9 and 40.0 per cent unique lines. While remaining (57%) lines in SPD method were paired with only 1 line, 60 per cent lines in SPDS method were paired with two lines (Table 4). BM had only 11.8 per cent unique lines while 64.7 per cent lines were paired with 4 or more lines.

In cross 6, SPD method had 66.7 per cent unique lines followed by PM with 58.8 per cent unique lines. BM was the poorest with only 13.3 per cent unique lines and 66.7 per cent lines pairing with 4 or more lines (Table 4).

Table 4. Comparison of breeding methods for producing unique lines in 6 crosses using SSR markers

Method	Unique lines (%)	Lines pairing with (%)			
		One line	Two lines	3 lines	≥4 lines
Cross 1 [(SL 525 x AGS 328) x {(SL 744 x SL 682) x AGS 752}]					
PM	16.7	16.7	0.0	0.0	66.7
SPDS	20.0	0.0	0.0	80.0	0.0
SPD	100.0	0.0	0.0	0.0	0.0
BM	26.7	0.0	33.3	20.0	20.0
Cross 2 [SL 525 x {(AGS 328 x SL 682) x AGS 752}]					
PM	9.1	9.1	18.2	27.3	36.4
SPDS	50.0	33.3	16.7	0.0	0.0
SPD	66.7	33.3	0.0	0.0	0.0
BM	33.3	16.7	50.0	0.0	0.0

Cross 3 (SL 744 x (SL 525 x AGS 328))					
PM	27.3	0.0	18.2	54.5	0.0
SPDS	57.1	0.0	42.9	0.0	0.0
SPD	100.0	0.0	0.0	0.0	0.0
BM	53.8	15.4	0.0	30.8	0.0
Cross 4 (SL 525 x IC 391477)					
PM	21.4	14.3	64.3	0.0	0.0
SPDS	20.0	80.0	0.0	0.0	0.0
SPD	50.0	0.0	25.0	25.0	0.0
BM	25.0	15.0	0.0	0.0	60.0
Cross 5 (SL 525 x Lsb 23)					
PM	23.1	15.4	0.0	0.0	61.5
SPDS	40.0	0.0	60.0	0.0	0.0
SPD	42.9	57.1	0.0	0.0	0.0
BM	11.8	0.0	0.0	23.5	64.7
Cross 6 (SL 755 x SL 794)					
PM	58.8	0.0	17.6	23.5	0.0
SPDS	20.0	0.0	0.0	80.0	0.0
SPD	66.7	0.0	33.3	0.0	0.0
BM	13.3	13.3	0.0	6.7	66.7

DISCUSSION

18 polymorphic primers were used to compare efficiency of 4 breeding methods in six crosses. MCCLEAN *et al.* (2012) applied linear-plateau model for all the possible pairs of genotypes using 46 SSR markers and suggested that 18 markers provided accurate diversity estimates and hence were adequate to determine genetic similarity in *Phaseolus vulgaris* L. Overall, SPD method had the highest per cent of unique lines among all the methods. Also, in all the crosses except cross 3, no line was paired with more than 2 lines in SPD method. PM and BM had the lowest number of unique lines. The level of redundancy in case of BM and PM was high as evident from high number of lines pairing with 4 or more lines. SPD method had the least redundancy, therefore it was able to maintain maximum variability. FUNADA *et al.* (2013) while comparing SPD, SPDS and BM also reported that the BM had the highest redundancy. More lines in BM were paired with multiple lines as compared to SPD and single seed decent (SSD) method.

KEIM *et al*. (1994) used RFLP markers to compare two different populations, one population was developed using the SSD method and a second population with different parents was used to evaluate the SPD method. They used the number of paired-comparisons at a given level of genetic similarity as their criterion, which is an alike in-state criterion. They reported that the SPD method was 18 per cent less efficient than the SSD method, at the $Sxy \geq 0.875$ level.

In bulk method, chances of redundancy are more because of improper sampling and variable fecundity of different plants. Therefore in BM, variability is reduced in advanced generations. In single pod descent method, single pod is selected from each plant and then bulked. The difference between BM and SPD is that in SPD, the effect of variable fecundity is

eliminated. Also, because only single pod is selected from each plant in SPD, size of seed bulk is small and thus chances of improper sampling are reduced. On the contrary, in BM each plant contributes many seeds that leads to redundancy of plants in segregating generations. Therefore, SPD method had retained higher variability than the BM. MUEHLBAUER *et al.* (1981) used computer simulation to compare the SSD method with BM. When they simulated the BM and assumed 50 seeds per plant, only 25 per cent of the original F_2 plants were represented at the F_6 level of inbreeding. KERVELLA and FOUILLOUX (1992) used computer simulation to evaluate the multiple-seed procedure and the BM. They reported that when the multiple-seed procedure was used and 10 seeds were sampled per plant using 100 plants to represent the population, 67 per cent of the original F_2 plants would not be represented in the F_5 generation. When the SPD method was used with two seeds per pod, 55 per cent of the original F_2 plants were not represented in the F_5 population. The computer simulations of both KERVELLA and FOUILLOUX (1992) and MUEHLBAUER *et al*. (1981) used an identical-by-descent criterion to determine the relative efficiencies of each genetic sampling method.

In the present study, four breeding methods were studied; one without any selection (SPD), one with natural selection only (BM) and two with artificial selection (PM and SPDS). Selection, both natural and artificial always results in reduced variability either due to competition among lines or fecundity (KEIM *et al.*, 1994). The results in the present study also indicated reduced variability in lines derived by PM and BM.

CONCLUSION

Based on the dissimilarity coefficients using SSR markers, number of unique lines in various breeding methods was calculated. SPD method had the highest per cent of unique lines among all the methods in different crosses. In five out of six crosses studied, no line was paired with more than 2 lines in SPD depicting low redundancy. PM and BM had the lowest number of unique lines. The level of redundancy in case of BM and PM was high as evident from high number of lines pairing with 4 or more lines. Choice of the best method depends on the savings of time and labour when advancing populations as well as retaining maximum variability in the population. Among the 4 breeding methods used, BM was the least labour intensive method. However, SPD method retained much higher levels of variability till later generations. The breeder has to consider among high variability retained by SPD or ease of harvest and seed processing of BM while deciding the best method to fit his breeding program.

Received, July 30th, 2020
Accepted September 10th, 2021

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PROCENA EFIKASNOSTI METODA OPLEMENJIVANJA KORIŠĆENJEM MOLEKULARNIH MARKERA U SOJI

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Izvod

Četiri metode oplemenjivanja tj. metod pedigrea (PM), potomstvo jedne mahune (SPD), potomstvo jedne mahune sa selekcijom (SPDS) i bulk metod (BM) upoređeni su za održavanje varijabilnosti u populaciji u naprednim generacijama korišćenjem SSR markera. Linije $F_{4:7}$ dobijene kroz različite metode oplemenjivanja iz šest različitih ukrštanja su procenjene za broj jedinstvenih linija zadržanih u svakoj metodi sa koeficijentom sličnosti $\geq$ 0,875. Osamnaest polimorfnih SSR markera je korišćeno za procenu koeficijenta sličnosti između linija u okviru metode oplemenjivanja u svakom ukrštanju. U svim ukrštanjima, SPD metoda je bila najbolja metoda u proizvodnji jedinstvenih linija sa opsegom od 42,9 do 100 %. SPD metoda je takođe imala najmanji broj linija koje se uparuju sa dve ili više linija. PM i BM su imali najmanji broj jedinstvenih linija u po tri ukrštanja, a takođe je maksimalni udeo linija proizvedenih ovim dvema metodama bio uparen sa četiri ili više linija. Dakle, SPD metoda je bila najefikasnija među ove četiri metode u zadržavanju varijabilnosti u populaciji, ali oplemenjivač mora da napravi izbor između visoke varijabilnosti i efikasnosti žetve i kvaliteta semena da bi izabrao najpogodniji metod oplemenjivanja.

Primljeno 30.VII.2020.
Odobreno 10.IX. 2021.