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Usefulness of Improved Crossing Methods for Hybridization to Develop Mapping Population for Seed Longevity in Soybean [*Glycine max* (L.) Merrill]

T. V. Naflath[1], S. Rajendra Prasad[2] and R. L. Ravikumar[3]
[1,2]Department of Seed Science and Technology, [3]Plant Biotechnology, College of Agriculture, UAS, GKVK, Bengaluru - 560 065
e-Mail : nafsaidali@gmail.com

Abstract

Seed longevity in soybean is a serious concern with respect to the availability of quality seeds for sowing in the next season. The identification of donors for seed longevity and QTL mapping for selection are the major concerns in soybean breeding for seed longevity. In the present study, the longevity of Local Black Soybean (LBS) was confirmed by evaluating black, green and yellow seed coat colour genotypes for longevity through accelerated ageing technique. Two contrasting genotypes for seed longevity *i.e.*, LBS which showed highest germination of 85 per cent and JS 93-05 which recorded low germination (6 %) after accelerated ageing of seeds, were selected as parental lines to develop mapping population. These two parental lines differ for several morphological traits *viz*., growth type (indeterminate *vs* determinate), leaf shape (pointed ovate *vs* lanceolate), plant height (medium *vs* short) and pod colour (yellow *vs* brown), making it easy for identification of true F_1 through morphological traits. The success of hybridization through conventional method of emasculation in the previous evening followed by hybridization in the next day morning is very low. Hence, hybridization between JS 93-05 and LBS was carried out using two improved methods *i.e.*, wet cotton and Kolot method for comparison. The F_1 plants were grown in the field and in both the methods both selfed and true hybrids were observed morphologically. True F_1 plants resembled male parent for dominant traits like indeterminate growth, pointed ovate leaf, higher plant height and higher number of pods per plant. The true F_1 were confirmed through parental polymorphic SSR marker. Higher crossing success rate was recorded in wet cotton method (50 %) as compared to Kolot method (16.67 %). Totally, 400 F_1 seeds (green colour) were produced from confirmed F_1 plants to develop mapping population for seed longevity.

Keywords : Soybean, Seed longevity, Accelerated ageing, Hybridization, Mapping population

Seed longevity is a complex trait which is controlled by many factors both internally and externally. This is an important trait to preserve the genetic resources through dry seeds. Seed longevity is a major problem in soybean seeds, since the seeds lose their vigour and viability rapidly during storage especially at higher temperature and relative humidity conditions like in India (Singh and Ram, 1986). Hence, crop improvement programmes are necessary to develop cultivars resisting to the deteriorative changes occurring during storage by identifying genotypes with higher seed longevity (Dargahi *et al.,* 2014). Advances in genome sequencing and marker development have enabled highly specific and targeted breeding around important Quantitative Trait Locus (QTL) (Holloway & Li, 2010 and Uday & Hittalmani, 2016). Researchers have identified markers which are linked to the QTL through QTL mapping studies based on the recombination frequency for the given marker genes (Boopathi, 2020). A biparental mapping population is developed by using two genotypes which are contrasting for the trait of interest.

Soybean [*Glycine max* (L.) Merrill] flower is complete and perfect with papilionaceous flower structure. The androecium and gynoecium are covered by keel petal which ensures the self-pollination in soybean preventing the exposure of stigma to external pollen grains. Also, the elongated stamens form a ring around the stigma one day before pollination (Anonymous, 1996). Such a flower structure, small size and fragileness of

flower makes the soybean hybridization and crop improvement a tedious and skilful task along with the risk of flower drop. In the commonly followed method of crossing in soybean *i.e.*, hand emasculation in the previous day evening of the buds followed by pollination on the next day morning, the success rate observed is around 2-3 per cent (Fehr, 1980 and Agarwal *et al.*, 2001). The low success rate of hybridization is a limitation in soybean breeding.

To increase the success rate in hybridization different approaches are followed for hybridization *viz*., pollination without emasculation (Walker *et al.,* 1979 and Talukdar & Shivakumar, 2012), emasculation followed by crossing in the same day (Kolot, 1981), emasculating and pollinating around 4-6 pm (Weidong *et al*., 1996), wet cotton method (Agarwal *et al*., 2001) and use of gametocide (Lal *et al*., 2004). In the present study two improved methods, *i.e.,* emasculation followed by pollination in the same day (Kolot, 1981) and wet cotton method (Agarwal *et al*., 2001) were compared for the success in hybridization to develop a biparental mapping population after identifying the contrasting parents for seed longevity. These two methods emphasize on the prevention of stigma dryness which is a serious concern in successful pollination of soybean under Indian conditions.

Material and Methods

Selection of Contrasting Parental Genotypes for Seed Longevity for Hybridization

It has been reported earlier that black seed coat colour genotypes in general have higher longevity (Kuchlan *et al.,* 2010; Pawar *et al*., 2017 and Adsul *et al*., 2018). In order to confirm the seed longevity of the soybean genotypes with black seed coat colour the following thirteen genotypes with different seed coat colour were selected for the hybridization to develop mapping population.

The genotypes selected for the study are Local Black Soybean (LBS), Kalitur, Accession number 101 (ACCNo.101), ACCNo.109 and ACCNo.369 with black seed coat, JS 90-41 and 104-31 with green seed coat colour and JS 335, MAUS-2, JS 93-05, KHSB-2, MAUS-71 and MAUS-81 with yellow seed coat. The selected genotypes were evaluated for the seed longevity by following accelerated ageing method (Anonymous, 2010) at National Seed Project, University of Agricultural Sciences, Bangalore.

Accelerated Ageing : The freshly harvested seeds were used for the study. Forty two gram seeds of each genotype were placed in an ageing box with a wire mesh screen as specified by International Seed Testing Association (ISTA) (Anonymous, 2010) for soybean. The box was filled with 40 ml of distilled water and it was sealed all around to maintain more than 95 per cent relative humidity. The box was kept in an ageing chamber which maintained a constant temperature of 41 ± 0.3 ^{0}C. Seeds were subjected to ageing for 72 hour in ageing chamber. After the treatment seeds were weighed and kept for germination.

The laboratory germination test was carried out as per the ISTA rules (Anonymous, 1996) using between paper method. One hundred seeds in four replications for each genotype were allowed to germinate at temperature of 30 ^{0}C with a relative humidity of 90 ± 2 per cent up to five days. The germination counts were recorded on 5th day (first count) and per cent germination was expressed on normal seedling basis. Another set of seeds of all the genotypes were kept in the ageing box at room temperature without ageing treatment as control.

Statistical Analysis

Analysis of variance (One-way ANOVA) of the recorded germination data was done using SPSS software version *IBM SPSS 23*.

Hybridization

Based on the results of the first experiment, two contrasting genotypes for seed longevity were selected for the hybridization. The salient features of the selected parental ones are given below ;

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JS 93-05 (female parent) : Short plant, determinate growth, semi-erect habit, lanceolate leaf shape, purple flower colour, brown pod with pubescence, bold large seed with yellow seed coat colour.

Local Black Soybean (LBS) (male parent) : Medium plant, indeterminate growth, semi-erect growth type, pointed ovate leaf shape, purple flower colour, yellow colour pod with pubescence, medium sized seed with black seed coat colour.

The two genotypes were grown in pots in the greenhouse during *kharif* 2020. At flowering, the unopened swollen proximal flower buds (colour of petals just started appearing) which were about to open in next day morning in JS 93-05 were selected for emasculation. The remaining immature buds and self-pollinated flowers on that raceme were removed. After removing the calyx and corolla of the selected bud, the anther position was examined. If the ring of ten anthers below the stigma was not present then such buds were discarded. Also the anthers were observed for pollen dehiscence by taping on thumbnail. The bud was discarded after tapping if there was any deposition of yellow powder on thumbnail. Once the flower bud is selected, the anthers were removed without disturbing the stigma. Twenty emasculated flower buds each were used to operate two methods of hybridization.

Wet Cotton Method : Emasculation as mentioned earlier was done previous day evening (4-6 pm). Immediately after emasculation the stigma was covered with a water wet cotton (Fig. 2). Pollination was carried out in the next day morning between 9-11 a.m.

Kolot Method : Emasculation was conducted as explained above on the same day of pollination. Emasculation was done early in the morning between 6-7.30 a.m. followed by pollination at 9-11 a.m.

The crossed pods were harvested and seeds were collected. Six crossed seeds from each method were randomly selected and grown in the field along with parental lines to identify true hybrid plants using morphological contrasting traits of the parental genotypes.

Confirmation of True Hybrid using SSR Markers

The DNA was extracted from parental lines and all the field grown F_1 plants separately using CTAB method (Doyle and Doyle, 1990). The parental lines were screened with SSR markers to identify polymorphic SSR marker for screening the F_1 plants to confirm the true hybrid plants.

RESULTS AND DISCUSSION

Seed Longevity of Selected Genotypes

The germination per cent of genotypes without ageing treatment (control) was found to be non-significant and all the genotypes ranged from 99 to

JS 93-05

Local Black Soybean

Fig. 1. Seed germination after accelerated ageing treatment in JS 93-05 and Local Black Soybean

Fig. 2 : Water wet cotton covering emasculated flower bud

100 per cent germination. The genotypes showed significant difference for germination after accelerated ageing treatment (Fig. 1; Table 1). Highest germination per cent after ageing was found in the genotype LBS (85%) followed by ACC No.369 with 75 per cent. The lowest germination percentage was observed in JS 90-41 (2%) followed by 104-31 (3%), JS 93-05 (6%), JS-335 (7%).

TABLE 1
Germination percentage of soybean genotypes in control and accelerated ageing treatment

Genotypes	Germination (%)	
	Control	Accelerated ageing
JS-335	99.00	7.00 [a]
MAUS-2	100.00	9.00 [a]
JS 93-05	100.00	6.00 [a]
KHSB-2	99.00	20.00 [b]
MAUS-71	99.00	34.00 [c]
MAUS-81	100.00	48.00 [d]
JS 90-41	100.00	2.00 [a]
104-31	99.00	3.00 [a]
KALITUR	99.00	69.00 [e]
Local Black Soybean	100.00	85.00 [f]
ACCNo.101	99.00	65.00 [e]
ACCNo.109	99.00	73.00 [e]
ACCNo.369	100.00	75.00 [e]
CD (5 %)	NS	8.641

Significant genotypic variability in reduction of germination percentage after accelerated ageing treatment was also reported by Shruthi and Siddaraju (2018) in soybean. The black seed coat colour genotypes had higher per cent of germination when compared to yellow and green seed coat colour genotypes. The results are in agreement with the previous results observed by Pawar *et al.* (2017) and Adsul *et al*. (2018) in soybean. Kuchlan *et al*. (2010) reported that, the black seed coat colour varieties had lesser gap between the seed coat and cotyledon, fewer pores on the seed coat surface and higher lignin content in the seed coat, which makes them less susceptible to mechanical damage and deteriorative changes during ageing, than the genotypes with other seed coat colours.

Seed longevity is a major problem in soybean seed production and supply. All the cultivated varieties grown in India do not possess seed longevity for longer period. The genotypes with black seed colour are distinct from the cultivated varieties for longevity. It is important to transfer this trait to cultivated varieties. Seed longevity is a complex trait, therefore development of molecular markers linked to seed longevity will be useful for the transfer of targeted trait. Development of biparental mapping populations is an important step in mapping QTLs. In this direction the contrasting parental lines, the black seed coat colour Local Black Soybean variety as a donor male parent and JS 93-05 as female parent were selected for hybridization using two different methods.

Hybridization

In both the methods of hybridization the pod set was observed. The pod set after crossing was more in Kolot method as compared to wet cotton method with 35 and 25 per cent, respectively (Table 2). The flower drop is a major concern in soybean. Even under normal conditions of growth, the proportion of flowers developing in to mature pods ranges from 20 to 70 per cent depending on the environment and kind of seed (Huff and Dybing, 1979). The pollination process lowers the water

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TABLE 2

Success rate of pod set in two methods of hybridization in soybean

	Wet cotton method	%	Kolot method	%
Pod set	5.00	25.00	7.00	35.00
Flower drop	15.00	75.00	13.00	65.00
Total number of seeds	9.00		15.00	
Number of seeds per pod	1.80		2.14	
True cross out of 6 seeds	3.00	50.00	1.00	16.67

potential of the floral parts and increases its ability to absorb water (Huff and Dybing, 1979). Thus the external applications of the water may be essential for better pod development. However, in the present study the pod set percentage was lower in wet cotton method. This could be due to disturbance to the flower bud and stigma while covering the stigma and flower bud with wet cotton. It is important to take proper care not to disturb the flower buds while placing the wet cotton after emasculation as well as during pollination to reduce the flower drop in this method. The number of seeds per pod produced in wet cotton method (1.80) was relatively less compared to Kolot method (2.14). Only nine seeds were produced from 20 crossed flowers while 15 seeds were produced from the same number of flowers attempted in Kolot method. In wet cotton method the female flowers were handled two times, once during emasculation in the previous evening and second time for pollination in the next day. Such disturbances to small delicate flower buds may result in more number of flower drop and reduced seed set per pod. On the other hand in Kolot method the emasculation and pollination is done on the same day with reduced handling of the flower buds results in higher pod set and higher seed number per pod.

Identification of True F_1

The identification of true F_1 plants was carried out by carefully observing morphological traits of individual F_1 plants derived from both the methods. True F_1 plants of both the method of crossing showed indeterminate growth habit with pointed ovate leaves similar to male parent (Fig. 3). The mean plant height and number of pods per plant of true F_1 plants in wet cotton method was 52.20 cm and 48.33, respectively, while in the F_1 plants produced through Kolot method, it was 50.50 cm and 43.00,

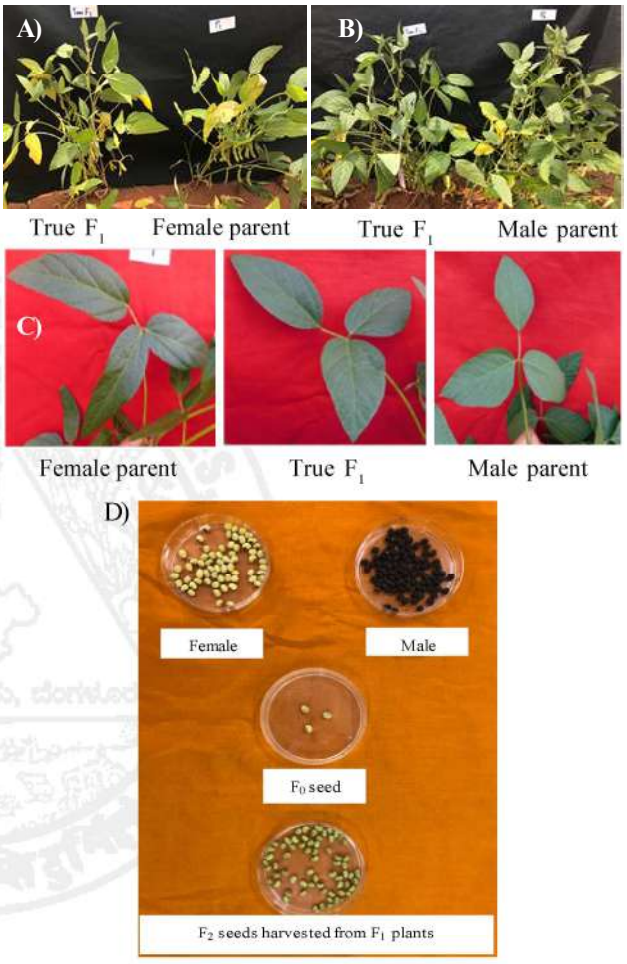

Fig. 3: Morphological comparison of soybean plants : A) F_1 vs female parent, B) F_1 vs male parent, C) Leaf shapes of parental lines and F_1, D) Seeds of female, male parent, F_0 and F_2 seeds harvested from F_1 plants

respectively. Indeterminate growth type, pointed ovate leaf shape, higher plant height, more number of pods per plant and presence of pod pubescence were observed in all the true F_1 plants resembling male parent. Caviness and Prongsirivathana (1968) reported that the plant height is governed by a single dominant gene with tallness being the dominant character. The growth habit of the plants appeared to be monogenically controlled with

dominance of indeterminate habit (Raut *et al*., 1994). The narrow leaf shape (lanceolate) in soybean is governed by a single recessive gene (*ln*) with dominance of ovate shape (Porter, 2000). Both additive and non-additive gene action were significantly involved in the expression of number of pods per plant (Thakare *et al.,* 2017). The seeds identified as selfed in both the methods resembled the female parent for all the morphological character (Table 3).

The hybridity of the F_1 plants was further confirmed by the colour of the seeds produced by true F_1 plants. The true F_1 plants produced green colour seeds (Fig. 3). The colour of the seeds obtained from crossed pods were yellow. The genetics of seed coat colour is complex. In soybean it was found that the yellow seed coat colour was dominant over black with a monogenic rate of 3:1 (yellow : black) in F_2 of a cross of Ankur x EC 30196 with yellow and black seed coat, respectively on the other hand, when a yellow coloured MACS-38 crossed with black coloured EC 30196, the F_2 segregation ratio was found to be 12:3:1 confirming the presence of epistatic gene interaction where yellow colour is epistatic to black (Raut *et al.,* 1994).

In the limited number of flowers used for hybridization, the success rate was more in wet cotton method (50.00% true hybrid seeds) compared to Kolot method (16.67%). Similar observations were made by Agarwal *et al.* (2001) with 33.57 per cent pod set and an average of 1.99 seeds per pod with wet cotton method compared to traditional and Kolot methods. The probable reason for higher success rate of the wet cotton method in producing true F_1 seeds could be due to the prevention of stigma dryness. Tilton *et al.* (1984) observed that the stigmas of soybean are wet and papillate. Shivakumar *et al*. (2016) reported that the stigma of the soybean is very delicate, highly susceptible to handling and becomes dry when exposed to external environment. Wet cotton covering helps to maintain humidity, reduce transpiration loss and also hastens the microclimate of the emasculated bud which would help to recover the buds from mechanical injury (Agarwal *et al*., 2001). The wet cotton covering also avoid the chance pollination by unknown pollen since it acts as a physical barrier. The less crossing success in case of Kolot method may be due to the differences in maturity of the stigma and anther while conducting pollination. Sometimes the pellicle layer of the stigma will not rupture which acts as a barrier for pollen entry, the non-availability of full bloom in male plant at the time of pollination are the drawback in Kolot method.

The major objective of the hybridization in the present study was to develop mapping population segregating for many quantitative traits and seed longevity. The parents are diverse and differ for many qualitative traits related to productivity (Table 3). We wanted to conform the hybridity of the true hybrid plants using molecular markers to increase the

TABLE 3

Morphological observations of parental lines and true F_1 plants of soybean

Traits	Female	Male	F_1 Wet cotton method	F_1 Kolot method	Selfed plants
Seed colour	Yellow	Black	Yellow	Yellow	Yellow
Growth type	Determinate	Indeterminate	Indeterminate	Indeterminate	Determinate
Leaf shape	Lanceolate	Pointed ovate	Pointed ovate	Pointed ovate	Lanceolate
Flower colour	Purple	Purple	Purple	Purple	Purple
Plant height (cm)	32.94	56.74	52.20	50.50	28.34
Pod pubescence	Present	Present	Present	Present	Present
Pod colour	Brown	Yellow	Yellow	Yellow	Brown
No. of pods/plant	25.00	52.80	48.33	43.00	21.40
Harvested Seed colour	Yellow	Black	Green	Green	Yellow

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reliability of mapping population. Out of the twenty five SSR markers used to identify the polymorphism between parental lines, only one SSR marker (BARCSOYSSR_19_1324; forward primer sequence- $^{5'}$TCTCTTTTCACGGTGGCTTC$^{3'}$ and reverse primer sequence- $^{5'}$TCTCTTTTCACGG TGGCTTC$^{3'}$) produced polymorphic bands between parental lines. The female parent produced an amplicon size of 180 bp while the male parent produced 170 bp band. The extent of polymorphism reported in soybean is very less (Singh *et al*., 2008).

The polymorphic SSR marker was used to genotype the hybrid plants (Fig. 4). All the true F_1 plants identified based on morphological traits produced double bands, thus confirm the hybridity of the plants (Talukdar and Shivakumar, 2012). In the present study the true F_1 plants were heterozygous with both the amplicons, while the selfed plants showed only one band similar to female parent.

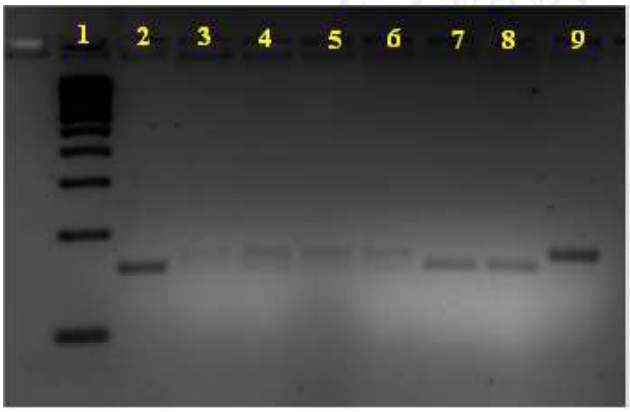

Fig. 4: Hybridity testing of soybean plants through SSR marker (BARCSOYSSR_19_1324). Lane 1: Ladder; Lane 2: Female parent; Lane 3-6: True F_1; Lane 7, 8: Selfed plant; Lane 9: Male parent

Since the soybean hybridization process is a challenging task, any method which gives higher success rate has a significance in the breeding programme. In the present study, wet cotton method produced higher crossing success rate than Kolot method. Further, in wet cotton method the chance pollination by the unknown pollen is also avoided since the stigma is covered with wet cotton which acts as a barrier. This method is very simple and easy to practice. However, the results are based on only one combination of two parental lines with small number of flowers for crossing. It is important to involve more number of diverse parental lines and across the seasons to confirm the results. From all the confirmed true F_1 plants, 400 seeds were produced which will be used for mapping the seed longevity trait by growing the next generation and also to forward them to develop Recombinant Inbred Lines (RILs) through single seed decent (SSD) method.

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