RESEARCH

Ensuring Genetic Purity of Pigeonpea Hybrids by Incorporating a Naked-Eye Polymorphic Marker in A and B Lines

K. B. Saxena, M. I. Vales,* R. V. Kumar, R. Sultana, and R. K. Srivastava

ABSTRACT

To enhance the productivity of pigeonpea [*Cajanus cajan* (L.) Millspaugh] a hybrid breeding technology, based on the cytoplasmic nuclear male-sterility (CMS) system and partial natural outcrossing, is currently been used. However, there are difficulties to maintain genetic purity of the hybrids and their parents. The incorporation of an easily identifiable morphological marker (naked eye polymorphism [NEP]) could be used to determine seed purity. The morphological marker selected for this study, obcordate leaf, is not present in cultivated pigeonpea; it is inherited as a single recessive gene and can be observed soon after planting (approx. 6 wk). To incorporate the obcordate leaf shape into hybrid parents, the trait was transferred from the germplasm accession ICP 5529 into male-sterile (A lines) and the corresponding maintainers (B lines). The hybrids derived from crosses involving obcordate leaf A lines and normal leaf fertility restorers (R lines) were fully fertile and had normal lanceolate leaves; thus the difference between A line and hybrids was clear. The use of obcordate leaf as a NEP marker in pigeonpea would contribute to preserve parental line purity and confirm hybrid status.

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, 502 324, A.P., India. Received 19 Nov. 2010. *Corresponding author (i.vales@cgiar.org).

Abbreviations: CMS, cytoplasmic nuclear male-sterility; NEP, naked eye polymorphism; SSR, simple sequence repeat.

To break the decades-old low yield plateau in pigeonpea [*Cajanus cajan* (L.) Millspaugh], plant breeders have recently developed a hybrid breeding technology (Saxena, 2008). This technology is primarily based on partial natural outcrossing (Saxena et al., 1990) and a cytoplasmic nuclear male-sterility (CMS) system (Saxena et al., 2005). Pigeonpea hybrids have shown potential for high seed yield in both multilocation as well as on-farm trials. In these trials, a medium maturing hybrid, ICPH 2671, exhibited yield advantages of 30% or more in seven pigeonpea growing states of India (Saxena and Nadarajan, 2010). In a number of such trials, hybrid reached 3.0 to 4.4 t ha^{-1}; this represent a high yield potential and a breakthrough compared with the historical stagnant yields (700–800 kg ha^{-1}). The high yields observed in pigeonpea hybrids could greatly contribute to increase production without increasing the area under production; this would be especially important for Indian agriculture, which, at the time, depends on pigeonpea importation to meet the national protein needs of the vastly vegetarian and resource poor masses.

Wind pollination in pigeonpea is extremely low or absent (Kumar and Saxena, 2001) and natural outcrossing is caused by a variety of pollinating insects (Williams, 1977; Onim, 1981). The extent of natural outcrossing is inconsistent across environments (Saxena et al., 1990) and, consequently, the recommended isolation distances to maintain pure stocks and develop hybrids are also

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quite variable. The natural outcrossing that is essential for pod setting on male-sterile plants to produce seeds is also responsible for genetic contamination of parental lines, if the production plots are not well isolated. In the absence of any firm recommendation on isolation specifications for different agro-ecological conditions, the problem of genetic contamination has become a potential threat to hybrid breeding programs and it can occur both at the parent and hybrid seed production levels.

To ensure consistency in the performance of hybrids it is important that genetic purity is maintained year after year while producing large seed quantities of hybrids and their parents. In most field crops, seed quality and germplasm identity is ensured by evaluating freshly harvested seed of hybrids and parents through the processes of standard grow-out tests. During the last 3 yr, it has been observed that maintenance of seed purity is a serious concern in pigeonpea hybrids due to its long generation turnover time that may extend from 6 to 9 mo. In addition, the inherent photoperiod sensitivity of the crop inhibits flowering of the plants in the off-season sowings. This further limits the grow-out options for quality determination. Therefore, efforts were made to identify and incorporate an easily identifiable morphological marker (naked eye polymorphism [NEP]) in A (male sterile) and the corresponding B (maintainer) parental lines to track purity of the inbred lines and corresponding hybrids for large scale commercial hybrid seed production. The exploration of the germplasm pool for NEPs (Table 1) led to the identification of obcordate leaf morphological marker present in accession ICP 5529. This morphological trait is absent in currently cultivated pigeonpea varieties; it is expressed in about 6 wk after sowing and easily can be assessed visually. In this paper we describe the inheritance of the obcordate leaf trait and the breeding and selection scheme followed at ICRISAT to incorporate this NEP leaf trait into A and B lines.

MATERIALS AND METHODS

To undertake this research the germplasm line ICP 5529 was selected. ICP 5529 was acquired by ICRISAT Gene Bank on 16 May 1973 from Kanpur, India, with alternate accession identifier numbers P 4864-1 and T 105. ICP 5529, an FAO in Trust Accession, is a nondeterminate medium maturing line with seeds of medium size (100 seed weight of 8 g) and brown color. The ICRISAT's Gene Bank records indicate that ICP 5529 has obtuse leaves. The emerging seedlings of ICP 5529 have a pair of lanceolate simple cotyledon leaves; the subsequent leaves are trifoliate and obtuse initially (Fig. 1) and become obcordate (most distinctive from normal lanceolate leaves) in about 6 wk from planting. It is also important to point out that the central leaflet expresses the obcordate leaf shape better than the lateral leaflets (Fig. 1). ICP 5529 could be related (even though there is no conclusive evidence) with a likely mutant plant having obcordate leaves and distinctive flower architecture found in 1939 in the Gorakhpur district of Uttar Pradesh, India, collected and brought to Kanpur where it was described tentatively as a different species, *Cajanus obcordifolia* Singh (Singh et al., 1942). This accession is currently maintained at ICRISAT's genebank as *Cajanus cajan*.

Information on the inheritance of the obcordate leaf trait and fertility restoration ability of the obcordate line ICP 5529 was essential for designing an effective breeding scheme. To achieve this, crosses were made between four A lines (ICPA 2089, ICPA 2047, ICPA 2048, and ICPA 2049) and the obcordate accession (ICP 5529). Fertility of F_1 plants was based on the assessment of pollen production in the fully grown buds at flowering and seed set. For inheritance studies of obcordate leaf trait, the F_1s and F_2s derived from the cross of ICP 5529 (male) and the A lines were inspected for leaf shape 6 wk after sowing. The test cross seeds were produced by crossing the F_1 of cross ICPA 2048 × ICP 5529 with obcordate parents. A goodness of fit Chi-square test was performed to check if the segregation fit a 3:1 ratio (segregation for a single recessive gene) in the F_2 generation and if the segregation fit a 1:1 ratio (also single recessive gene) in the BC_1F_1 generation.

Incorporation of the obcordate leaf trait was done separately for A lines (set I) and for B lines (set II). The original A lines (male-sterile) had the genotype A_4/rrNN, the original B lines (maintainer) had the genotype C_c/rrNN, and the R lines (restorer) had the genotype C_c/RRNN. The obcordate leaf donor ICP 5529 had the genotype C_c/RRnn. The breeding schemes to incorporate the obcordate leaf trait into A and B lines are included in the results section. A_4 represents sterile cytoplasm from *Cajanus cajanifolius,* while C_c represents fertile cytoplasm from *Cajanus cajan*; *r* represents male-sterility (recessive allele); *R* represents male-fertility (dominant allele); *N* represents normal lanceolate leaf (dominant *Llt* allele, compiled by Saxena et al., 2000); and *n* represents obcordate leaf (recessive *llt*^{o} allele, modified from Saxena et al., 2000) (Fig. 2 and 3).

RESULTS

Fertility Restoration of the Obcordate Leaf Donor Parent

All the F_1 plants of the four crosses between A lines and the obcordate leaf donor (ICP 5529) were fully male-fertile and had normal leaves (Table 2), suggesting that the obcordate leaf trait was recessive and that fertility restoration was due to the effect of dominant gene(s). Based on the information generated earlier on F_2s (using the same female lines) the presence of one or two dominant genes controlling fertility restoration of the male-sterile lines was reported (Dalvi et al., 2008; Saxena et al., 2011).

Inheritance of the Obcordate Leaf Trait

All the F_1 plants in the four crosses between A lines and the obcordate leaf donor (ICP 5529) had normal lanceolate leaves (Table 2), suggesting recessive nature of the obcordate trait. In the F_2 populations, segregation for leaf type fit well the expected ratio of three normal lanceolate leaves to 1 obcordate leaves (Table 2). Over all the four crosses, out of 1721 F_2 plants studied, 1301 had normal leaves and 420 plants were with obcordate leaves; this fit the expected ratio of three plants with normal lanceolate leaves to one plant with obcordate leaves, suggesting that the obcordate leaf

Table 1. Naked eye polymorphic (NEP) markers with potential to be used to track purity of pigeonpea hybrids and their corresponding parental lines; their expression time and inheritance.

Trait	Phenotype	Genetic control†	Stage of expression	Heritability
Stem color	purple vs. green	dominant, single gene	6–8 wk after sowing	low
Leaf shape	obcordate leaf vs. normal	recessive, single gene	6 wk after sowing	high
Leaf width	narrow leaf vs. broad leaf	recessive, single gene	4–5 wk after sowing	high
Growth habit	determinate vs. nondeterminate	recessive, single gene	flowering	high
Flower color	yellow vs. red	recessive, oligo-genes	flowering	low–moderate
Pod color	green vs. purple	recessive, oligo-genes	4 wk after flowering	low–moderate
Seed color	white vs. brown	recessive, oligo-genes	maturity	moderate

†For more details and references see Saxena and Sharma (1990).

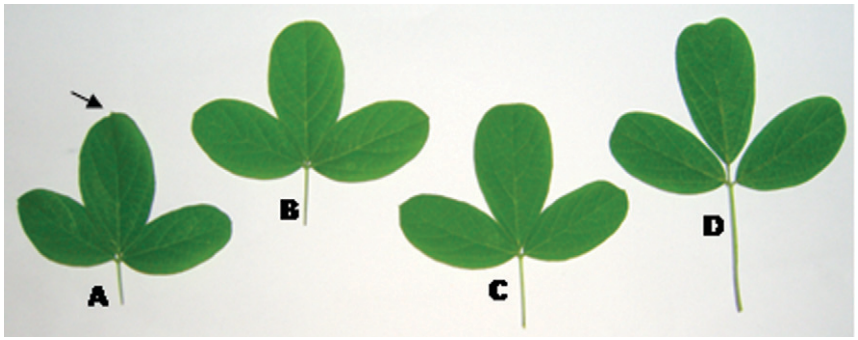

Figure 1. Transition of obtuse to obcordate leaflets in ICP 5529. The arrow points to the terminal leaflet transitioning from obtuse (A) to obovate (B), truncate (C), and finally into obcordate (D); all of them are clearly different from normal lanceolate leaves.

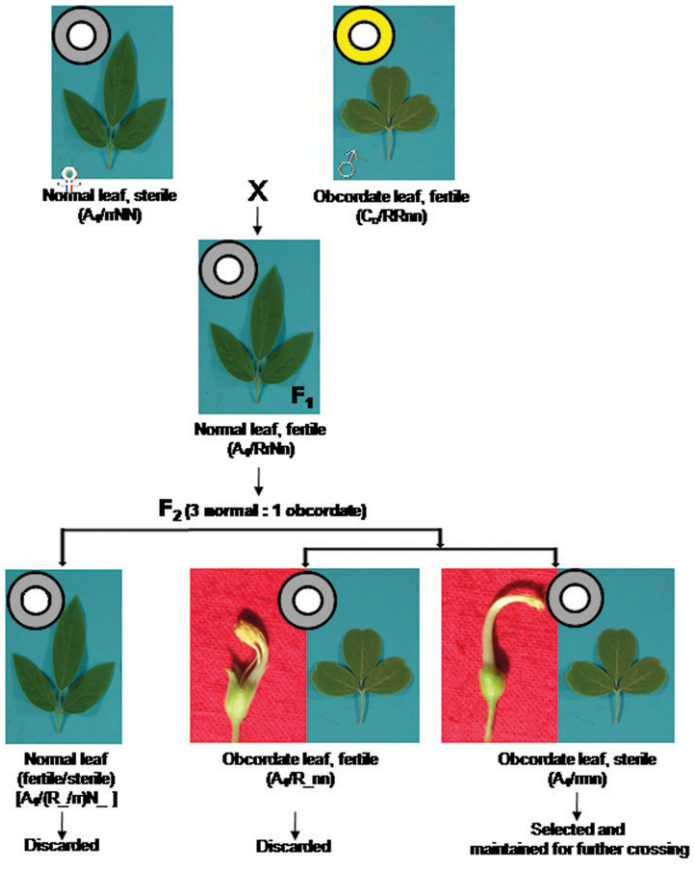

Figure 2. Breeding scheme to introgress the obcordate leaf trait from ICP 5529 to generate A lines (A_4/rrnn) with the obcordate leaves (set I).

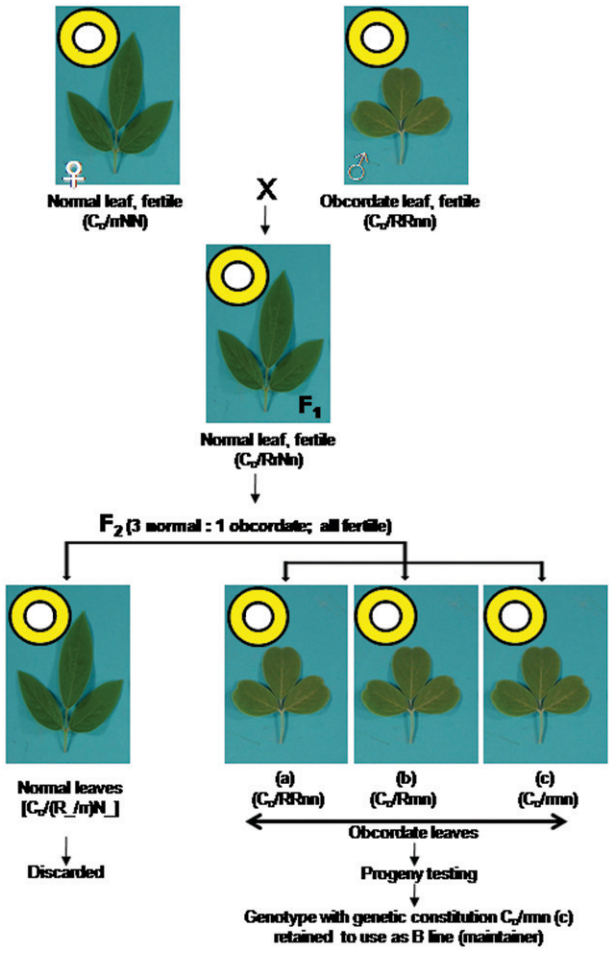

Figure 3. Breeding scheme to introgress the obcordate leaf trait from the ICP 5529 to generate B lines (C_c/rrnn) with the obcordate leaf trait (set II).

Table 2. Phenotype of leaf and male-fertility in F_1 and segregation for leaf type in F_2 and BC_1F_1 generations.

Cross	No. of F_1s	No. of F_2 plants: Phenotype of F_1s leaf type and male-fertility	Total	Normal leaf	Obcordate leaf	χ^2 (3:1)	*p* value
ICPA 2089 × ICP 5529	4	Normal and fertile	99	79	20	1.22	0.27
ICPA 2047 × ICP 5529	41	Normal and fertile	549	408	141	0.14	0.71
ICPA 2048 × ICP 5529	60	Normal and fertile	518	396	122	0.58	0.45
ICPA 2049 × ICP 5529	35	Normal and fertile	555	418	137	0.03	0.86
Total	140	–	1721	1301	420	0.33	0.57
						(1:1)	
BC_1 F_1 plants (ICPA 2048 × ICP 5529) × ICP5529			116	61	55	0.31	0.58

trait was controlled by a single recessive gene ($p = 0.57$). In the BC_1F_1 generation ([ICPA 2048 × ICP 5529] × ICP 5529), 61 and 55 plants, respectively, had lanceolate and obcordate leaves (Table 2). This data fit the expected ratio of 1:1 ($p = 0.58$), confirming that the obcordate leaf trait was controlled by a single recessive gene. Single plant data combining fertility and leaf shape traits was not collected in F_2 plants; thus it was not possible to test the independent assortment of fertility restoration and leaf morphology. However, A (male sterile) and B (male fertile) lines with obcordate leaves were successfully produced.

Breeding Scheme to Incorporate the Obcordate Leaf Marker in A and B Lines

A scheme to develop obcordate CMS lines A lines (set I) and their corresponding B lines (set II) was launched at ICRISAT in 2006. To breed male-sterile (A lines) and its corresponding maintainer (B lines) with obcordate leaves, it was essential to preserve the male-sterility–inducing cytoplasm base of *Cajanus cajanifolius* in the A lines and its fertility maintaining cytoplasm in the B lines. The breeding program involved two important traits (obcordate leaf and male-sterility) controlled by recessive alleles; thus the hybridization and selection scheme was designed and implemented very carefully. To achieve the target, breeding of A (set I) and B (set II) lines was undertaken separately.

Set I – Development of A Lines with Obcordate Leaves (A_4/rrnn)

To develop breeding lines with the obcordate leaf trait, A lines ICPA 2047 and ICPA 2048 (genotype A_4/rrNN) with normal leaf shape were crossed to the obcordate leaf donor ICP 5529 (C_c/RRnn). The F_1 seeds obtained from crosses were grown in a glasshouse in the off-season. In the F_1 generation, all plants were fertile with normal leaf shape (A_4/RrNn) (Fig. 2; Table 2). These plants were selfed to produce F_2 seeds. In 2007 season, F_2 populations were sown in field and male-sterile plants with obcordate leaves (A4/rrnn genotype) were selected. In the F_2 generation, plants with normal leaves (A_4/R_N_ and A_4/rrN_) and obcordate leaves (A_4/R_nn and A_4/rrnn) were observed.

The phenotypic segregation observed in F_2 generation was 1301 plants with normal leaves and 420 plants with obcordate leaves from a total of 1721 plants. From this population, all normal leaf plants were discarded at the seedling stage; the remaining plants with obcordate leaves were maintained. At flowering, all fertile plants with obcordate leaf with genotypic constitution of A_4/R_nn were removed and only male-sterile plants with obcordate leaves having genetic constitution A_4/rrnn were retained and selected for further crossing (Fig. 2).

Set II – Development of B Lines with Obcordate Leaves (C_c/rrnn)

Following a similar procedure as described above, B lines (C_c/rrNN) ICPB 2047 and ICPB 2048 with normal leaf shape were crossed with the obcordate leaf donor ICP 5529 (C_c/RRnn). In the F_1 generation, all plants were fertile with normal leaf shape (C_c/RrNn). These plants were selfed to produce F_2 seeds. All the F_2 seedlings with normal leaves (C_c/R_N_ and C_c/rrN_), inspected 6 wk after planting, were discarded. The rest of the F_2 plants with obcordate leaves were allowed to grow. Finally, C_c/rrnn B line type plants were selected through progeny testing (Fig. 3). To achieve this, obcordate leaf type of plants were sampled randomly and crossed with the male-sterile obcordate selections (A_4/rrnn) obtained from set I. Crosses with *a* type (C_c/RRnn) of plants produced all fertile plants (C_c/Rrnn) with obcordate leaves and therefore were rejected. The F_1 plants from crosses involving *b* (C_c/Rrnn) type of plants segregated in 50:50% fertile obcordate (C_c/Rrnn):male-sterile obcordate (C_c/rrnn) and were also rejected. On the other hand, the crosses made with *c* type (C_c/rrnn) of plants produced only male-sterile plants with obcordate leaves (A_4/rrnn), and such pollinator plants were selected and retained to be used as B lines (maintainers) to pollinate A line/s (obtained in set I). The progenies of such combination will produce all male-sterile plants with obcordate leaves, while the progeny of B lines produced fertile obcordate leaf plants.

The genotypes selected from sets I and II had recessive alleles both for obcordate leaf and male-sterility traits but their cytoplasm were different: A_4, *C. cajanifolius* cytoplasm

Table 3. Segregation for leaf shape and fertility restoration in hybrids (A × R) and B and R lines.

		Number of plants				
			Obcordate leaf		Normal leaf	
Category	Pedigree†	Total	MS‡	MF	MS	MF
A × B	A 2047-1-5 × B 2047-1-5	138	138	0	0	0
B	B 2047-1-5 (self progeny)	176	0	176	0	0
A × R	A 2047-1-5 × ICPL 87119 (R)	44	0	0	0	44
R	ICPL 87119 (R self progeny)	16	0	0	0	16
A × R	A 2047-1-5 × ICPL 20107 (R)	17	0	0	0	17
R	ICPL 20107 (R self progeny)	17	0	0	0	17
A × B	A 2047-2-3 × B 2047-2-3	138	138	0	0	0
B	B 2047-2-3 (self progeny)	194	0	194	0	0
A × R	A 2047-2-3 × ICPL 20096 (R)	55	0	0	0	55
R	ICPL 20096 (R self progeny)	26	0	0	0	26
A × R	A 2047-2-3 × ICPL 20108 (R)	32	0	0	0	32
R	ICPL 20108 (R self progeny)	21	0	0	0	21
A × R	A 2047-2-3 × ICPL 20111 (R)	3	0	0	0	3
R	ICPL 20111 (R self progeny)	24	0	0	0	24
A × B	A 2048-6-2 × B 2048-6-2	106	106	0	0	0
B	B 2048-6-2 (self progeny)	196	0	196	0	0
A × R	A 2048-6-2 × ICPL 87119 (R)	11	0	0	0	11
R	ICPL 87119 (R self progeny)	24	0	0	0	24
A × R	A 2048-6-2 × ICPL 20108 (R)	11	0	0	0	11
R	ICPL 20108 (R self progeny)	28	0	0	0	28
A × B	A 2048-14-2 × B 2048-14-2	115	115	0	0	0
B	B 2048-14-2 (self progeny)	316	0	316	0	0
A × R	A 2048-14-2 × ICPL 20096 (R)	18	0	0	0	18
R	ICPL 20096 (R self progeny)	31	0	0	0	31
A × R	A 2048-14-2 × ICPL 20098 (R)	18	0	0	0	18
R	ICPL 20098 (R self progeny)	19	0	0	0	19
A × R	A 2048-14-2 × ICPL 20107 (R)	14	0	0	0	14
R	ICPL 20107 (R self progeny)	26	0	0	0	26
A × B	A 2047-15-3 × B 2047-15-3	90	90	0	0	0
B	B 2047-15-3 (self progeny)	133	0	133	0	0
A × B	A 2047-14-2 × B 2047-14-2	91	91	0	0	0
B	B 2047-14-2 (self progeny)	153	0	153	0	0
A × B	A 2048-11-2 × B 2048-11-2	93	93	0	0	0
B	B 2048-11-2 (self progeny)	138	0	138	0	0
A × B	A 2048-14-2 × B 2048-14-2	47	47	0	0	0
B	B 2048-14-2 (self progeny)	127	0	127	0	0
	Total (A × B)	*818*	*818*	*0*	*0*	*0*
	Total (B-self progenies)	*1,433*	*0*	*1,433*	*0*	*0*
	Total (A × R)	*223*	*0*	*0*	*0*	*223*
	Total (R-self progenies)	*232*	*0*	*0*	*0*	*232*

†A, ICPA; B, ICPB.

‡MS: male sterile; MF: male fertile.

in set I (A lines) vs. C_c, *C. cajan* cytoplasm in set II (B lines). Hence, in set I all the plants selected were male-sterile while in set II all the plants selected were male-fertile due to their normal (fertile) cytoplasm. The obcordate plants from A and B type selections when crossed produced only obcordate male-sterile plants and B types plants produced all fertile obcordate leaf plants.

To verify the breeding behavior of the selections made in set I and II, the newly bred A lines were crossed with corresponding B lines. The F_1s were grown along with self B lines. The observations recorded in the progenies showed a perfect segregation for leaf type (Table 3). In the progenies of eight A × B crosses, all 818 plants were male-sterile and had obcordate leaves; while selfing of B type selections produced all (1433) male-fertile plants with obcordate leaves. This verification process was further extended to 10 hybrids by crossing obcordate male-sterile lines with known fertility restorers (R). All the progenies of these (A × R) crosses were male-fertile and had normal lanceolate leaves (Table 3), while the R lines bred true with respect to

lanceolate leaves. The presence of obcordate leaves does not seem to affect agronomic, disease resistances, and quality traits based on preliminary comparisons (data not shown) between obcordate vs. normal plants.

DISCUSSION

Soon after the development of stable CMS systems in pigeonpea (Saxena et al., 2005), the breeding of commercial hybrids started gaining momentum in India. The pigeonpea hybrid breeding technology is now inching toward perfection and a few high-yielding hybrids are in final stages of on-farm and marketing trials (Saxena and Nadarajan, 2010). To derive maximum benefit from this new technology, it is imperative that the purity of the hybrids is maintained year after year. One of the problems that the seed producers are facing at present is the maintenance of seed quality of hybrids and their parents. This will influence the performance of the hybrids with respect to uniformity, stability of fertility restoration, and specific trait maintenance. From a commercial point of view also, it is very important for sustainability of hybrids in the market. Hence, it is essential to assess the level of hybrid purity in each lot of hybrid seeds before it is released for marketing. Ideally, such quality determinations should be performed by seed producers using grow-out tests that involve growing the hybrid (A × R) and parent seeds and observing the plants for any morphological marker present only in the pollinator (R or B) line. Such marker traits should be easy to identify, cost effective, and less time consuming. Since most pigeonpea cultivars are of late maturity and the traditional grow-out tests would take a long time, and for quick results the options available are the use of molecular or morphological markers that are stable, easy to apply, and suitable for large on-farm scale use. Among the morphological markers, the obcordate leaf trait was selected because it fits the criteria indicated above and gives results within 6 wk of planting.

Application of the Grow-Out Test

The segregation data of the progenies showed that the selection for breeding obcordate A and B lines was successful. The progenies of crosses involving A and B lines were obcordate and male-sterile, while the self progenies of B lines were fertile and had obcordate leaves. These lines will be further stabilized by backcrossing before using them in the hybrid breeding programs. These materials can also be used to breed diverse CMS lines with obcordate leaves. In contrast, all the hybrid plants, produced using obcordate A lines, had normal lanceolate leaves.

Since obcordate leaf shape is a rare recessive trait and is not present in any cultivar presently grown in India or elsewhere, the presence of plants with normal lanceolate leaves in the seed production plots of the female parent (A line) will indicate contamination due to unwanted outcrossing caused by pollen from the plants with normal lanceolate leaves or to unexpected selfing of the A lines due to partial recovery of male-fertility. Since there is no seed dormancy in pigeonpea, the genetic contamination of the A lines can be detected by sowing freshly harvested seed from the female parent in pots at any time of the year and observing the seedlings for leaf shape. The seedlings emerging from the contaminated seeds will have normal lanceolate leaves and can be accurately counted within a short period of 6 wk. This way, a large number of parental seed lots can be examined for quality very quickly. In a similar way, sowing freshly harvested hybrid seed and inspecting leaf shape 6 wk after planting will allow to differentiate hybrids (normal lanceolate leaves) seed from sib mating (A × B lines). This system will not allow differentiating hybrids vs. R (restorer) plants, but since hybrid seed is only harvested from the maternal A plants, the contamination of hybrid seed with self R seed is less likely unless seed from R plants is harvested and mixed accidentally with hybrid seed.

As an alternate approach, simple sequence repeat (SSR) markers suitable for determining the identity and purity of pigeonpea hybrids and corresponding parents have been recently described and practically applied. The purity of a pigeonpea hybrid ICPH 2438 seed lots from two sources was successfully assessed and two SSR markers, CCB4 and CCttc006, were found as the most suitable to determine purity assessment (Saxena et al., 2010). This represents an excellent contribution for practical breeding and commercial seed production; applying this technology to large scale on-farm operation would require the establishment of formal seed sampling and submission of materials to an approved laboratory to perform DNA purity tests. This should be feasible, but other options such as the use of a NEP are worth exploring to provide alternatives to farmers and seed companies. If the A lines have the distinctive obcordate leaf shape and the R lines have normal lanceolate leaves, the hybrids (A × R) will have normal lanceolate leaves. Thus, in addition to verify the identity of the hybrids it will also be easy to differentiate the A and B lines from the hybrids. The use of NEP for hybrids and parental lines purity testing is very cost effective, quick, and can be easily implemented even by resource-poor seed growing farmers because it only depends on visual assessments, and in addition it is weather proof and does not require special facilities.

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