

Development of Cytoplasmic–Nuclear Male Sterility, Its Inheritance, and Potential Use in Hybrid Pigeonpea Breeding

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Pigeonpea [*Cajanus cajan* (L.) Millsp.] is a unique food legume because of its partial (20–30%) outcrossing nature, which provides an opportunity to breed commercial hybrids. To achieve this, it is essential to have a stable male-sterility system. This paper reports the selection of a cytoplasmic–nuclear male-sterility (CMS) system derived from an interspecific cross between a wild relative of pigeonpea (*Cajanus sericeus* Benth. ex. Bak.) and a cultivar. This male-sterility source was used to breed agronomically superior CMS lines in early (ICPA 2068), medium (ICPA 2032), and late (ICPA 2030) maturity durations. Twenty-three fertility restorers and 30 male-sterility maintainers were selected to develop genetically diverse hybrid combinations. Histological studies revealed that vacuolation of growing tetrads and persistence of tetrad wall were primary causes of the manifestation of male sterility. Genetic studies showed that 2 dominant genes, of which one had inhibitory gene action, controlled fertility restoration in the hybrids. The experimental hybrids such as TK 030003 and TK 030009 in early, ICPH 2307 and TK 030625 in medium, and TK 030861 and TK 030851 in late maturity groups exhibited 30–88% standard heterosis in multi-location trials.

Key words: *Cajanus sericeus*, cytoplasmic–nuclear male sterility, fertility restorers, hybrids, male-sterility maintainers, pigeonpea

Cajanus cajan (L.) Millsp., pigeonpea, is an important food legume of tropics and subtropics. To overcome the problem of yield stagnation in pigeonpea, a hybrid breeding program based on insect-aided natural outcrossing and genetic male sterility (GMS) was initiated at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). Six hybrids with 35–60% yield advantages over popular varieties were

released in India (Saxena 2002). These hybrids, however, failed due to various cost- and quality-related constraints that are inherently associated with any GMS system. To overcome these bottlenecks, breeding of a stable cytoplasmic–nuclear male-sterility (CMS) system was required (Ariyanayagam et al. 1995; Tikka et al. 1997; Wanjari et al. 2001; Saxena and Kumar 2003; Saxena et al. 2005). The male-sterile genotype selected by Ariyanayagam et al. (1995) from the cross involving *Cajanus sericeus* Benth. ex Bak., a wild relative of pigeonpea and a cultivar, looked promising but the expression of male sterility was not stable from one season to another (Saxena et al. 1996). The authors, therefore, selected this material for further breeding to develop an improved CMS system. This paper reports the development of CMS lines, its histology, inheritance of fertility restoration, and discusses the prospects for breeding commercial pigeonpea hybrids.

Materials and Methods

Development and Characterization of Stable CMS System

One of the male-sterile selections bred by Ariyanayagam et al. (1995) with 93% pollen sterility and reasonable biomass was selected for further breeding. This male-sterile plant was crossed with cultivar ICPL 85010. The F₁ seed was planted in a 25-cm diameter plastic pot filled with farmyard manure and Alfisol in 4:1 ratio. To determine the pollen fertility of this plant, 16 fully grown floral buds were harvested randomly and their anthers were squashed in 2% aceto-carmin solution. In each slide, brightly stained pollen grains were counted in 3–4 microscopic fields. This plant was backcrossed with ICPL 85010, and 8 BC₁F₁ seeds were produced. The seeds were sown on 23 July 1996 in a glasshouse. In order to diversify the genetic base, 7 BC₁F₁

male-sterile plants were crossed with 21 diverse inbred lines and their F_1 hybrids were grown in field. Of the 147 hybrids sown, 9 expressed >90% male sterility. From this group, 3 parents, one each in early (ICPL 88034), medium (ICPL 99044), and late (ICPL 366) maturity durations, were selected as recurrent parents for developing new sets of backcross materials. All the backcross (BC_1F_1 to BC_6F_1) progenies were grown in field. For large-scale seed multiplication, the BC_6F_1 seeds and their recurrent parents (maintainers B lines) were planted in different isolations on 18 June 2002 in 6 sets, each consisting of 10-m-long rows, in 4 male-sterile (A) lines:1 maintainer (B) line ratio. The seed was produced through insect pollinations. For morphological characterization, in each set 5 competitive plants were randomly selected, and data were recorded on days to flower, plant height (cm), seed color, growth habit, and 100-seed mass (g). Data from the 6 sets were pooled to estimate mean and standard error of each trait. For cytological studies of meiocytes, immature floral buds were fixed in Carnoy's II solution (acetic acid 1:chloroform 3:ethanol 6) for 24 h at 4 °C and then transferred to Carnoy's I solution (acetic acid 1:ethanol 3). Transverse sections of 3–5 μ m thickness were cut using a rotary microtome.

Search for Fertility Restorers

To identify fertility restorers and male-sterility maintainers, 251 advanced breeding and germplasm lines were crossed with ICPA 2032 and their F_1 s were evaluated for 3 consecutive years. Based on their pollen fertility, each line was classified either as restorer, maintainer, or segregating type. To study the genetics of fertility restoration, ICPA 2067 was crossed with a known restorer line ICP 12320. The F_1 plants were selfed with musclin cloth bags and also backcrossed to the male-sterile line. The parents, F_1 , F_2 , and BC_1F_1 populations were grown in field during 2007 rainy season. Data on segregation for male sterility and fertility were recorded in each generation. Chi-square test was applied for testing goodness of fit for each phenotypic ratio.

Evaluation of Hybrids

In 2005 rainy season (June to September), 10 medium-duration hybrids were produced at ICRISAT by crossing ICPA 2032 with known fertility restorers. The hybrids were evaluated at 5 locations in 2 replications during 2006 rainy season. The seed of CMS lines was also shared with Maharashtra Hybrid Seed Company (MAHYCO) and they produced 14 early-, 12 medium-, and 16 late-duration experimental hybrids. These hybrids were also evaluated in the same season at 6 locations with 3 replications. Each entry in both ICRISAT and MAHYCO trials was sown in a 4-m long 4-row plot. The sowings were done on ridges, 75 cm apart with plant-to-plant spacing of 20 cm. A basal fertilizer dose of di-ammonium phosphate was applied at 100 kg/ha to provide 18 kg N and 46 kg P. For controlling weeds preemergence, herbicide Basalin at 2 kg/ha was applied followed by 2–3 hand weedings. The trials were irrigated 2–3 times. Endosulfan (active ingredient) at 1.5 l/ha was sprayed 4 times during flowering and podding stages for

Table 1 Segregation for male sterility in F_1 and backcross generations in 3 crosses

Year	Generation	Male-sterile plants (%)		
		ICPA 2068 (ICPL 88034)	ICPA 2032 (ICPL 99044)	ICPA 2030 (ICPL 366)
1996	F_1	95 (222)	96 (18)	98 (32)
1997	BC_1F_1	97 (1700)	94 (111)	96 (35)
1998	BC_2F_1	99 (3275)	97 (237)	99 (87)
1999	BC_3F_1	91 (904)	98 (488)	96 (231)
2000	BC_4F_1	96 (5183)	98 (287)	99 (108)
2001	BC_5F_1	98 (3003)	99 (221)	100 (163)
2002	BC_6F_1	98 (2118)	97 (1239)	100 (1010)

Number of plants are given in parentheses.

effective control of the pod borer *Helicoverpa armigera*. In each plot, data were recorded for days to maturity, grain yield, plant height, 100-seed mass, and grain yield. Only yield data are reported.

Results and Discussion

Development of CMS Lines

Cajanus sericeus (Benth. ex. Baker) van der Maesen comb. nov., formally known as *Atylosia sericea* (Benth. ex. Baker) or *Cantharospermum sericeum* (Benth. ex. Baker), is a short-lived perennial bush endemic to deciduous monsoon forests of western Ghats and Satpura mountains of India (van der Maesen 1986). This wild relative of pigeonpea with $2n = 2x = 22$ is rich in protein (Saxena et al. 2002), and it can be crossed with cultivated pigeonpea (20–22% protein). The pod set on the selected male-sterile segregant from the material generated by Ariyanayagam et al. (1995) was low, and from 60 pollinations, only one pod with a single F_1 seed was harvested that yielded a male-sterile plant with shriveled anthers and no pollen grains. In BC_1F_1 generation, pod set was not good, and from 170 pollinations, only 3 pods were harvested that yielded only 8 seeds. The problem of low pod set, however, was overcome in subsequent generations. Out of 8 BC_1F_1 plants available, 7 had no pollen grains and 1 plant exhibited 48% pollen fertility. Out of 21 hybrids produced on the male-sterile plants, 9 exhibited 90–98% male sterility. From this group of maintainers, only 3 cross combinations involving ICPL 88034 (early maturity) with 95% male sterility, ICPL 99044 (medium maturity) with 96% male sterility, and ICPL 366 (late maturity) with 98% male sterility were selected (Table 1) for further backcrossing. In the remaining hybrids, the proportions of male-sterile plants varied from 35–60%. The BC_6F_1 population of ICPL 88034 with 98% male sterility was designated as ICPA 2068. Similarly, the backcross populations of ICPL 99044 (97% male sterility) and ICPL 366 (100% male sterility) were respectively designated as ICPA 2032 and ICPA 2030.

The assessment of A and B lines indicated that there were no marked differences between the 2 types for key morphological traits. Anthers of A lines were shriveled and light yellow with no pollen grains. The plants of all 3

male-sterile lines were nondeterminate with green stem and yellow flower color with red veins. ICPA 2068 and ICPA 2032 were semispreading in growth habit whereas ICPA 2030 was compact. The plants of ICPA 2068 were 130.8 ± 3.92 cm in height and flowered in 77.9 ± 0.61 days. The green-colored pods with dense purple streaks, on average, produced 3.20 ± 0.03 seeds. The seeds of ICPA 2068 were round and brown with 100-seed mass of 10.9 ± 0.15 g. ICPA 2032 flowered in 124 ± 1.13 days and were 220 ± 4.86 cm. It is a white-seeded A line with 100-seed mass of 11.3 ± 0.21 g. ICPA 2032 was resistant to both wilt and sterility mosaic diseases. The late maturing A-line ICPA 2030 was tall (240 ± 5.97 cm) and flowered in 145 ± 1.81 days. Seeds were round shaped and brown with 100-seed mass of 11.6 ± 0.40 g.

Histological Studies

The histological studies revealed that meiosis in both male-fertile and male-sterile plants proceeded normally up to the tetrad stage (Figure 1a,b), and during this period, the tapetum remained intact (Figure 1c,d). The tetrads in male-sterile plants remained enclosed within the tetrad wall due to persistence of tetrad walls and, subsequently, it led to vacuolation and abortion of pollen grains without influencing female fertility of plants. On the contrary, microsporogenesis in male-fertile plants proceeded normally (Figure 1e,g). Although the pollen grains in male-sterile anthers lost their contents, they remained as tetrads (Figure 1f), and at the end of microsporogenesis, only empty anther lobes were present (Figure 1h). In this male sterile, the gene controlling male sterility started functioning at tetrad stage. The tapetum is known to play a major role in microsporogenesis by producing and transporting vital enzymes, hormones, and nutritive materials to pollen mother cells (Vasil 1967; Yogesha 1991; Chhabra et al. 1997). The reasons for degeneration of microspores in the present CMS material is completely different than those reported earlier in GMS pigeonpea lines where persistence of tapetum (Reddy et al. 1978), early breakdown of tapetum (Dundas et al. 1981), and abortion of pollen mother cells due to delayed breakdown of tapetum (Dundas et al. 1982) were responsible for the manifestation of male sterility. In the present study, the growth of tapetum layer in the male-sterile anthers was normal, but the tetrad wall did not dissolve to release pollen grains and remained persistent for a longer period. The inability of the microspores to absorb nutrients and/or the persistence of tetrad wall are likely reason(s) for the vacuolation of tetrads leading to the collapse of pollen mother cells. This has been reported in *Allium cepa* (Virnich 1967) and in an interspecific cross between *C. cajan* and *Cajanus acutifolius* (Mallikarjuna and Saxena 2005).

Male-Sterility Maintainers and Fertility Restorers

To produce heterotic hybrids for diverse environments, it is important to have ample genetic diversity in both male-sterile (A) and fertility restorer (R) lines. Therefore, to start the hybrid breeding program, a search for fertility restorers

and male-sterility maintainers was made in advanced breeding lines, cultivars, and other pigeonpea germplasm. Among 251 F_1 s evaluated, 30 (12.0%) maintained male sterility, 23 (9.2%) restored fertility, and 198 (78.9%) segregated for male-fertility and sterility traits. Fourteen lines in early (MN 1, ICPLs 2, 30, 86, 148, 156, 183, 184, 205, 87091, 88012, 88039, 89031, and 90013); 13 lines in medium (BKT-3, HPL 40, ICP 10948, ICPLs 5, 111, 149, 150, 161, 211, 262, 265, 271, and 96052); and three in late (ICPs 7035, 9145, and ICPL 96045) duration were quality maintainers.

The extent of male sterility among the maintainers ranged between 95 and 100%. These lines exhibited a considerable variation for flowering (45–135 days), maturity (95–200 days), plant height (60–225 cm), and 100-seed mass (8.3–17.8 g). Among these, ICPLs 271, 90013, 96045, 96052, ICPs 7035, 9145, and 10948 were resistant to both fusarium wilt and sterility mosaic diseases whereas 7 lines were resistant to sterility mosaic disease only. The A lines derived from these maintainers through backcrossing can be used to develop hybrids for disease-prone areas. Similarly, the A lines derived from ICPL 88039 and MN-1 can be used to breed early-maturing hybrids.

Among 22 fertility restorers identified, 12 were of early (ICP 4406, ICPLs 12, 20, 89, 118, 129, 205, 83006, 83009, 90001, 90004, and 99011); 5 of medium (HPLs 21, 24, ICP 6997, and ICPLs 131 and 277); and 5 of late (ICPs 10650, 11912, 11376, 12320, and 13991) maturity groups. These lines also showed considerable variation for agronomic traits such as days to flower (68–177 days), plant height (98–220 cm), and 100-seed mass (7.4–15.3 g). Among the restorers, HPL 21, HPL 24, ICP 6997, and ICP 11376 had resistance to sterility mosaic disease. In the present study, a majority (78.9%) of the F_1 hybrids exhibited noticeable variation for pollen fertility. In pigeonpea, where a considerable level of heterozygosity exists within germplasm accessions, the chances of getting such segregations are always high. This variation could also be due to differential inter-genomic or cytoplasmic-genomic interactions. Such interactions usually involve complex genetic phenomenon such as complementation, inhibition, epistasis, etc., which render the fertility restoration highly fragile. In addition, specific environmental factors such as temperature and photoperiod also influence pollen fertility/sterility of plants (Kaul 1988). According to Abdalla and Hermesen (1972), the polymorphism, arising due to differential gene actions, also results in inconsistent expression of male fertility.

Genetic Studies

In genetic studies, all 35 F_1 plants of hybrid ICPA 2067 \times ICP 12320 were male fertile indicating the dominance of fertility restoring genes. As expected, the F_2 and BC_1F_1 populations of this cross segregated for male sterility and fertility. Out of 359 F_2 plants grown, 303 were fertile where as only 56 exhibited male sterility. This segregation fit well to a ratio of 13 fertile:3 sterile ($P = 0.01$). In BC_1F_1 generation out of 175 plants, 121 were male fertile and 54

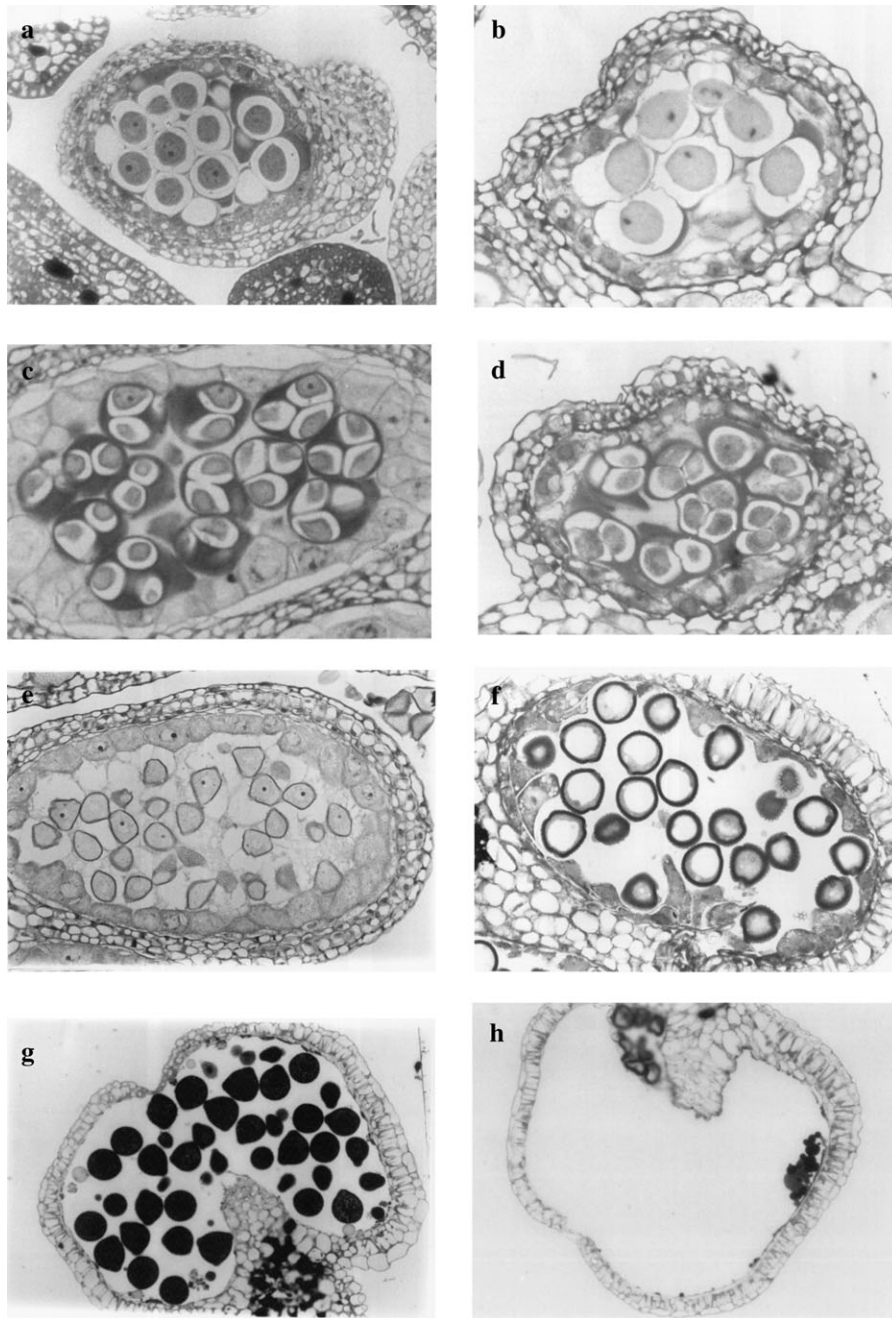


Figure 1. Transverse sections of flower buds at various stages of microsporogenesis in male-fertile (a,c,e,g) and male-sterile line (b,d,f,h).

had male-sterile anthers, which showed a good fit for a 3 fertile:1 sterile ($P = 0.01$) ratio. These results suggested the presence of 2 dominant genes, with one basic and one inhibitory gene action for the determination of fertility restoration in ICPA 2067. In pearl millet, 1–3 dominant genes (Yadav DV, submitted) and in *Vicia faba* 1–2 dominant genes (Kaul 1988) governed the fertility restoration. In the present experiment, only one cross was studied; the conclusions about fertility restoration cannot be generalized. Additional studies are necessary to fully

understand the genetic systems underlying the fertility restoration of this male-sterility system.

Hybrid Vigor for Grain Yield

Although pigeonpea breeding activity began in the early part of 20th century, the first report of hybrid vigor in the crop was published by Solomon et al. (1957). In 10 intervarietal crosses, they reported up to 24.5% superiority over the best parent for grain yield. They recorded significant hybrid vigor

Table 2 Mean yield (kg/ha) of pigeonpea hybrids at 5 locations in central India, during 2004 rainy season

Hybrid no.	Locations					Mean	% Superiority over control
	Nagpur	Ravalcol	Patancheru	Manoharabad	Jalna		
ICPH 2307	4196	2727	2342	1852	2819	2787	40.1
ICPH 2899	2872	3038	2187	1667	1484	2250	13.1
ICPH 2308	3205	1951	2159	2037	1069	2084	4.7
ICPH 2337	3125	2117	2231	1574	1252	2060	3.5
ICPH 2900	3371	2514	1646	1111	1511	2031	2.1
Asha (C)	2372	2389	2256	1481	1425	1990	—
ICPH 2336	2641	2008	1918	1574	1652	1959	—
ICPH 2305	2314	2491	2247	1574	1113	1948	—
ICPH 2897	3028	1601	2256	1389	1363	1927	—
ICPH 2334	2708	1666	1975	1667	1334	1870	—
ICPH 2898	2664	1875	2245	1019	1244	1809	—
Mean	2954.2	2216.1	2132.9	1540.5	1478.7	2064.5	
Standard error of the mean	±363.0	±227.6	±296.4	±131.7	±138.4	±231.4	
Coefficient of variance (%)	16.7	15.1	19.9	12.3	14.4	15.7	

for plant height, plant spread, stem girth, number of fruiting branches, and leaf length and width. Subsequently, a number of reports were published on hybrid vigor for yield and yield components in pigeonpea (Saxena and Sharma 1990). The GMS-based hybrids exhibited 35–60% heterosis for yield (Saxena 2005). Kalaimagal and Ravikesavan (2003) reported heterosis ranging from 10% to 58% and 10% to 106% over better parent and control variety, respectively. Kandalkar (2007) found that CMS-based hybrids recorded standard heterosis up to 156% for grain yield; Saxena (2007) reported

yield advantage of 50–100%. Chauhan et al. (2008) using a line × tester design reported 19.9–26.1% heterosis for yield, and they attributed it to increased number of pods per plant, pod length, and seed size. In multilocation yield trials, the differences among entries were significant at all 5 locations (Table 2). Out of 10 hybrids evaluated, only ICPH 2307 performed well across the locations. This hybrid recorded 2787 kg/ha yield with 40% superiority over control variety Asha. In Nagpur, ICPH 2307 produced 4196 kg/ha yield, and it was 77% superior to the control. This hybrid recorded

Table 3 Yield (kg/ha) of some selected short-, medium-, and long-duration hybrids in multilocation trials conducted at 6 locations by MAHYCO, during 2004 rainy season

Hybrid no.	Locations						Mean	% Superiority over control
	Jalna	Sarwadi	Yeotmal	Gulbarga	Shmshabad	Khargone		
Short duration								
TK 030003	1319	1264	2111	2340	1250	2417	1781	87.9
TK 030009	1625	1354	1964	1604	1291	1618	1576	66.2
TK 030006	1250	1598	1971	1382	1319	1424	1491	57.3
ICPL 88039 (C)	1041	917	1278	792	868	792	948	—
Standard error of the mean (SEM)	±180.4	±291.5	±163.1	±94.7	±257.8	±99.6	±181.2	—
Coefficient of variance (CV, %)	17.4	16.5	15.8	7.5	13.7	7.8	13.1	—
Medium duration								
TK 030625	2281	3267	2310	2945	2319	2978	2683	74.2
TK 040174	2270	2890	2198	2688	2423	2750	2537	64.7
TK 030555	2104	2820	2180	2653	2034	2835	2438	58.3
BSMR 736 (C)	1180	2145	1284	1750	1320	1550	1540	—
SEM	±79.9	±99.4	±125.1	±118.4	±95.7	±522.2	±173.5	—
CV (%)	4.8	4.1	7.4	5.5	5.1	7.0	5.7	—
Late duration								
TK 030861	2386	2741	2867	3435	3085	2778	2882	46.7
TK 030851	2595	2472	3017	3115	2867	2640	2784	41.8
TK 030811	2614	1695	2545	3550	3166	1737	2551	29.9
ICPL 87119 (C)	1945	2082	1756	2061	1784	2153	1964	—
SEM	±386.1	±158.1	±280.0	±307.8	±212.4	±131.6	±246.0	—
CV (%)	21.8	9.7	17.8	13.9	11.1	6.9	13.5	—

14%, 25%, and 98% standard heterosis at Ravalcol, Manoharabad, and Jalna, respectively.

MAHYCO, the largest private seed company in India, was the first to adopt ICRISAT's hybrid pigeonpea technology, and their hybrids in early-, medium-, and late-duration groups produced good results (Table 3). In early maturity, hybrid TK 30003 recorded yield of 1781 kg/ha with 87.9% superiority over the control. In Khargone, this hybrid produced 2417 kg/ha yield with 205% superiority over the control. In medium-duration group, hybrid TK 030625 gave highest yield (2683 kg/ha) with 74.2% superiority over the control. Among late-maturity group, TK 030861 was the best hybrid with mean yield of 2882 kg/ha and 46.7% heterosis. This hybrid recorded 3435 kg/ha yield with 66.7% standard heterosis in Gulbarga. These results of the multilocation trials suggest that with the help of stable CMS lines and available insect-aided natural outcrossing, the heterosis could be exploited at commercial level in pigeonpea.

Hybrid Seed Production Technology

Because the extent of natural outcrossing in pigeonpea is determined by the number of pollinating insects, its extent also varies from one place to another (Saxena et al. 1990). Therefore, it is difficult to specify isolation distance that would be effective at every location. Saxena (2006), however, recommend an isolation distance of 500 m for quality hybrid seed production. For the production of breeders' seed of male-sterile line, A and B lines are grown in a row ratio of 4:1. In a seed multiplication plot of ICPA 2032, a total of 35 kg of crossed seed was harvested from 225 m² block with an estimated yield of 1555 kg/ha. For the production of hybrid seed, the A line and its pollen parent (R line) also are grown in 4:1 ratio. Using this technology in 2005 rainy season, from a small (120 m²) plot a total of 13-kg (=1083 kg/ha) hybrid seed was produced. Because the maintainer (B) and restorer (R) lines are male fertile in nature, their genetically pure seed can be produced without much difficulty in isolations.

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