Genetics of Fertility Restoration in A_4-Based, Diverse Maturing Hybrids of Pigeonpea [*Cajanus cajan* (L.) Millsp.]

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ABSTRACT

Recently, a breeding technology for hybrid pigeonpea [*Cajanus cajan* (L.) Millsp.] based on cytoplasmic-nuclear male sterility (CMS) and partial natural outcrossing was developed at ICRISAT. The limited number of experimental hybrids tested has shown the presence of a considerable amount of hybrid vigor for seed yield. In the present study, one extra-early- (120 d), two early- (150 d), and two late-maturing (180 d) pigeonpea hybrids were studied to generate information on the genetics of fertility restoration of the A_4 CMS system. In the extra-early-maturing hybrids, pollen fertility was controlled by a single dominant gene, whereas in the early- and late- maturing hybrids, male fertility was governed by two duplicate dominant genes. It was also observed that hybrids with two dominant genes produced a greater pollen load and expressed greater stability as compared with those carrying a single dominant gene. The information on the inheritance of fertility restoration will help in designing strategies for breeding elite hybrid parents, and it was concluded that for breeding hybrids with stable fertility restoration, the presence of two dominant genes is essential.

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Abbreviations: CMS, cytoplasmic-nuclear male sterility.

Although pigeonpea [*Cajanus cajan* (L.) Millsp.] is a crop of significance in various tropical and subtropical agricultural regions, it has suffered from yield stagnation for more than six decades (FAOSTAT, 2008). Pigeonpea, a high-protein food legume, is a partially outcrossed species (Saxena et al., 1990), but crop breeders in yield-enhancement programs ignored the potential consequences of that fact and unsuccessfully used the breeding methods traditionally recommended for a self-pollinating species (Singh et al., 2005; Saxena, 2008). In pigeonpea, natural outcrossing varies from 25 to 50%, and the pollen is carried by a number of pollinating insects. Studies at ICRISAT have shown that this level of outcrossing is sufficient to maintain cytoplasmic-nuclear male-sterility (CMS) lines and also to produce F_1 hybrid seeds. In the seed-production plots grown in isolation, we have repeatedly harvested more than 1000 kg ha^{-1} seeds. Commercial seed companies are now using this technology with a 4:1 female-to-male ratio.

Given the increasingly limited land available for agriculture and the continuously increasing population, the amount of protein available to the rural masses is gradually declining, particularly in Africa and South Asia. Therefore, enhancing pigeonpea yield has become a major concern to all stakeholders. To overcome this situation, ICRISAT took the initiative of developing commercial

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hybrids, an approach that has never been used with any food legume until now. To achieve this goal, the availability of a stable CMS system was essential. The initial breeding efforts in this direction were unsuccessful (Reddy and Faris, 1981), but a breakthrough was achieved by Saxena et al. (2005) when they bred the CMS line ICPA 2039, which is based on cytoplasm derived from *Cajanus cajanifolius* [(Hains) van der Measen comb. Nov], a wild relative of pigeonpea, and designated it as A_4. This CMS line was found to be stable across environments and had good ability to restore male fertility in hybrid combinations.

Because pigeonpea is cultivated under diverse environments and cropping systems with specific maturity and plant-type requirements (Saxena, 2008), the CMS trait from ICPA 2039 was transferred to extra-early (ICPA 2089), early (ICPA 2039), and late-maturing (ICPA 2043) lines to facilitate the development of hybrids in diverse maturity groups for different agroclimatic zones. Information on the genetics of fertility restoration helped in designing strategies for breeding elite hybrid parents, and this paper reports the genetics of fertility restoration in five crosses of three maturity groups.

MATERIALS AND METHODS

Our experience in the hybrid-breeding program for the past 3 yr has indicated that, in contrast to early- and late-maturing types, fertility restoration in extra-early-maturing hybrids was poor and highly erratic when tested across several locations. Therefore, as a first step to identify stable extra-early-maturing fertility restorers, ICPA 2089 was crossed with 11 inbreds of similar maturity, and the F_1 progeny were grown in 2008 at four locations: three in northern India (Gurdaspur, Faridkot, and Ludhiana, 30–32° N) and one in southern India (Patancheru,17° N). At each location, every F_1 plant (Table 1) was examined for pollen fertility. On the basis of the stability of fertility restoration, hybrid PHP 42 (ICPA 2089 × PHR 31) was selected for studying the inheritance of fertility restoration.

For that purpose, crosses were made within maturity groups. The extra-early A line (ICPA 2089) was crossed to PHR 31. The early-maturing A line (ICPA 2039) was crossed to both ICPR 2438 and ICPR 2447, while the late-maturing male-sterile line (ICPA 2043) was crossed to two restorer lines: ICPR 2671 and ICPR 3467 (Table 2). Segregation for fertility restoration was studied in the F_1, F_2, and BC_1F_1 generations in the extra-early and late-maturing crosses at Patancheru. In addition, in one late-maturing cross (ICPA 2043 × ICPR 3467), 53 fertile F_3 offspring were also assessed for segregation. In the early-maturing materials, backcross seed could not be produced due to severe damage caused by pod borers (*Maruca testulalis* Geyer.). For assessing pollen fertility, 10 fully grown but unopened floral buds were harvested from different parts of the plants between 9 and 11 a.m. Anthers from the sampled flowers were removed and squashed in 1% acetocarmine solution. Three microscopic fields on each slide were examined under the light microscope. Counts for fertile and sterile pollen grains were made, pollen grains being considered fertile if they were stained with dye (deep red color). Within each population, discrimination among the plants for male-fertility restorers and nonrestorers was done on the basis of their pollen-fertility data. Plants with more than 85% (mean of 10 observations) stained pollen grains were classified as male fertile, whereas those with less than 5% pollen fertility were considered male sterile. Only six plants were found to be partially male sterile, but on selfing, these plants also did not set pods and therefore were considered male sterile. The data thus obtained were subjected to chi-square analyses for testing their goodness of fit to different expected phenotypic ratios.

RESULTS

Extra-Early-Maturing Crosses

For inheritance studies of extra-early-maturing crosses, 11 F_1 hybrids were made with ICPA 2089 to identify good fertility restorers. Each hybrid was grown at four locations and examined for fertility restoration (Table 1). Significant effects of the location on the expression of pollen fertility were clear: the highest mean pollen fertility (65.2%) was recorded at Gurdaspur, whereas the lowest fertility restoration (37.9%) occurred at Ludhiana (Table 1). Pollen fertility of the hybrids at the other two locations was more or less similar (55.1 and 53.1% at Faridkot and Patancheru, respectively). The hybrid PHP 42 (ICPA 2089 × PHR 31) had the highest mean pollen fertility (83.8%) across the locations. This hybrid recorded 100 and 94.4% fertility restoration at Gurdaspur and Patancheru, respectively, and hence, was selected for inheritance study.

In the F_1 generation of the hybrid PHP 42, 17 of the 18 plants grown were male fertile, demonstrating the dominance of fertility restoration (Table 2). Visual observations on pollen load in the buds showed that in comparison to the inbred male parents, the fertile hybrid plants produced fewer pollen grains. The partially male-sterile plants were found to have pollen fertility between 20 and 50%, but the pollen grains were small. These partially male-sterile plants were also examined for pod set under controlled pollination (selfing), and only 4 plants produced any pods: one or two underdeveloped pods with one small, dark-colored seed in each pod. Consequently, such plants were considered male sterile for the chi-square analyses. The overall segregation for male sterility in the F_2 generation (115 fertile and 32 sterile) fit well with the expected ratio of 3 fertile to 1 sterile ($p = 0.37$). In the BC_1F_1 generation, the fertile and sterile segregants were almost equal in number and fit well with the expected 1:1 ratio ($p = 0.05$), confirming that a single dominant gene governed the pollen fertility in PHP 42.

Early-Maturing Crosses

Two early-maturing crosses were made with ICPA 2039, one with ICPR 2438 and the other with ICPR 2447 as the male parents. Both of these crosses behaved in a similar manner as far as the segregation of male fertility and

Table 1. Fertility restoration of 11 extra-early-maturing pigeonpea hybrids at four locations during the rainy season, 2009.

Hybrid	Parentage	Fertility restoration				
		Gurdaspur	Faridkot	Patancheru	Ludhiana	Mean
		%				
PHP 32	ICPA 2089 × PHR21	37.5 (16)†	30.8 (13)	35.0 (20)	21.7 (23)	31.3 (72)
PHP 33	ICPA 2089 × PHR22	60.0 (10)	50.0 (12)	55.6 (18)	50.0 (26)	53.9 (66)
PHP 34	ICPA 2089 × PHR23	66.7 (15)	73.3 (15)	75.0 (20)	47.6 (21)	65.7 (71)
PHP 35	ICPA 2089 × PHR24	37.5 (8)	56.3 (16)	63.2 (19)	36.4 (22)	48.3 (65)
PHP 36	ICPA 2089 × PHR25	60.0 (10)	33.3 (6)	30.0 (20)	23.1 (26)	36.6 (62)
PHP 37	ICPA 2089 × PHR26	83.3 (6)	60.0 (15)	31.6 (19)	29.4 (17)	51.1 (57)
PHP 38	ICPA 2089 × PHR27	83.3 (6)	56.3 (16)	68.4 (19)	26.1 (23)	58.5 (64)
PHP 39	ICPA 2089 × PHR28	42.9 (7)	57.1 (14)	47.4 (19)	58.8 (17)	51.5 (57)
PHP 40	ICPA 2089 × PHR29	71.4 (7)	55.6 (9)	27.8 (18)	43.5 (23)	49.6 (57)
PHP 41	ICPA 2089 × PHR30	75.0 (4)	62.5 (16)	55.6 (18)	10.5 (19)	50.9 (57)
PHP 42	ICPA 2089 × PHR31	100.0 (11)	70.6 (17)	94.4 (18)	70.0 (20)	83.8 (66)
Location mean		65.2 (100)	55.1 (149)	53.1 (208)	37.9 (237)	52.8 (694)

†Numbers in parentheses are numbers of plants.

Table 2. Segregation for male fertility and sterility in F_1, F_2, and BC_1F_1 generations and their chi-square tests.

Crosses	Generation	No. of plants			Expected ratio	χ^2 calculated†	Probability
		Total	Fertile	Sterile			
Extra-early-maturing hybrid							
ICPA 2089 × PHR 31	F_1	18	17	01	1:0	—	—
	F_2	147	115	32	3:1	0.82	0.37
	BC_1F_1	187	107	80	1:1	3.90	0.05
Early-maturing hybrids							
ICPA 2039 × ICPR 2438	F_1	175	175	0	1:0	—	—
	F_2	225	212	13	15:1	0.09	0.77
ICPA 2039 × ICPR 2447	F_1	170	170	0	1:0	—	—
	F_2	222	210	12	15:1	0.27	0.60
Late-maturing hybrids							
ICPA 2043 × ICPR 2671	F_1	138	138	0	1:0	—	—
	F_2	238	223	15	15:1	0.00	0.97
	BC_1F_1	138	98	40	3:1	1.17	0.28
ICPA 2043 × ICPR 3467	F_1	90	90	0	1:0	—	—
	F_2	230	216	14	15:1	0.01	0.92
	BC_1F_1	189	140	49	3:1	0.09	0.77

†For each generation, χ^2 calculated separately segregating for male fertility and sterility.

sterility was concerned. In the F_1 generation, dominance of male fertility was observed, whereas in F_2 generation, the populations segregated and fitted well with the expected digenic duplicate dominant ratio (Table 2) of 15 fertile to 1 sterile ($p = 0.60$ for cross 1 and $p = 0.77$ for cross 2).

Late-Maturing Crosses

Two late-maturing fertility restorers, ICPR 3467 and ICPR 2671, were crossed with ICPA 2043. The complete dominance of fertility-restoring genes was observed in the F_1 generation, where all the hybrid plants were fully fertile and had a good pollen load. In the F_2 generation, both of the hybrids exhibited a digenic ratio with a duplicate dominant (15:1 ratio) gene action. This hypothesis of gene action was confirmed in the BC_1F_1 generation of both of the crosses (Table 2), where the ratio of 3 fertile to 1 sterile was observed.

In the F_3 generation of cross ICPA 2043 × ICPR 3467, out of 96 random fertile progeny grown, 53 segregated for fertility or sterility (Table 3), whereas 43 did not segregate. This segregation pattern fitted well with the expected ratio of 8 segregating to 7 nonsegregating ($p = 0.72$). The variation observed within the 53 segregating progeny (Table 3) indicated the presence of two subgroups. Subgroup I, with 33 progeny, segregated in a dihybrid ratio of 15 fertile to 1 sterile (pooled $p = 0.94$), while in subgroup II (20 progeny) the progeny segregated only for one gene, resulting in a ration of 3 fertile to 1 sterile (pooled $p = 0.28$). Overall the two subgroups segregated in the expected ratio of 1:1 ($p = 0.07$).

Table 3. Segregation for male fertility and sterility in F_3 fertile progenies derived from the late-maturing cross ICPA 2043 × ICPR 3467.

Progeny number	Number of plants			χ^2 calculated†	Probability
	Total	Fertile	Sterile		
Group1 (15:1 ratio)					
3	35	34	1	0.69	0.41
5	22	21	1	0.11	0.74
8	31	29	2	0.00	0.96
9	31	29	2	0.00	0.96
16	33	32	1	0.58	0.44
20	36	35	1	0.74	0.39
22	29	28	1	0.39	0.53
23	35	34	1	0.69	0.41
24	20	18	2	0.48	0.49
25	31	30	1	0.48	0.49
27	37	35	2	0.05	0.83
29	19	17	2	0.59	0.44
30	19	17	2	0.59	0.44
31	31	29	2	0.00	0.96
34	33	31	2	0.00	0.96
35	34	31	3	0.38	0.54
42	28	27	1	0.34	0.56
47	34	32	2	0.01	0.93
50	33	32	1	0.58	0.44
53	30	27	3	0.72	0.40
54	28	27	1	0.34	0.56
55	18	17	1	0.01	0.90
56	16	15	1	0.00	1.00
58	29	26	3	0.83	0.36
59	23	22	1	0.14	0.71
63	18	16	2	0.73	0.39
67	30	27	3	0.72	0.40
72	24	22	2	0.18	0.67
73	18	17	1	0.01	0.90
79	14	13	1	0.02	0.89
89	18	16	2	0.73	0.39
90	22	21	1	0.11	0.74
95	29	26	3	0.83	0.36
Pooled (n = 33)	888	833	55	0.00	0.94
Group II (3:1 ratio)					
11	24	18	6	0.00	1.00
12	37	31	6	1.52	0.22
13	33	24	9	0.09	0.76
14	26	15	11	4.15	0.04
18	32	24	8	0.00	1.00
32	33	28	5	1.71	0.19
33	33	27	6	0.82	0.37
36	32	20	12	2.67	0.10
37	30	23	7	0.04	0.83
38	33	25	8	0.01	0.92
41	44	33	11	0.00	1.00
45	34	27	7	0.35	0.55
49	33	25	8	0.01	0.92
65	28	24	4	1.71	0.19
68	21	18	3	1.29	0.26
70	27	21	6	0.11	0.74
76	21	18	3	1.29	0.26
81	16	13	3	0.33	0.56
92	18	14	4	0.07	0.79
94	18	13	5	0.07	0.79
Pooled (n = 20)	573	441	132	1.18	0.28

†χ^2 calculated between two segregating groups (15:1 versus 3:1) for 1:1 ratio is 3.19 (p = 0.07).

DISCUSSION

In the present study three A lines with A_4 cytoplasm were crossed to five fertility-restoring lines to study the inheritance of male fertility and two male-fertility-restoring genes segregating independently. In the extra-early hybrid (PHP 42), the fertility restoration was imparted by a single dominant gene, whereas in the other four hybrids, two dominant genes were involved in the expression of fertility restoration with duplicate dominant gene action. From the present studies, it appeared that the visual differences observed in pollen yield among the hybrid plants were linked to the number of fertility-restoring genes present in an individual. In the hybrid plants with two fertility-restoring genes, the pollen load in the floral buds was similar to that of pure-line cultivars. In this context, it is worth mentioning that a late-maturing hybrid, ICPH 2671, which has two dominant fertility-restoring genes (Dalvi et al., 2008), has shown high stability of yield and fertility restoration in farmers' fields in seven diverse states of India and three provinces of Myanmar (Saxena and Nadrajan, 2010). On the contrary, when a single fertility-restoring gene was present in the hybrids, they produced relatively fewer pollen grains and their fertility restoration was unstable in diverse environments. Hughes and Bodden (1977) also identified certain wheat (*Triticum aestivum* L.) lines with poor pollen production, and they concluded that the restorer parents with a single gene were responsible for their poor pollinating capacity. Saxena et al. (1981) studied the cytology of a partially male-fertile line of pigeonpea and reported that in such plants the breakdown of the tapetum was irregular and that there was no consistency in the extent and the site of degeneration of the tapetal tissues in different flowers and plants. The microsporogenesis of the present partially male-fertile plants of pigeonpea was not examined in this study, but it is likely that both pollen development and its release processes were defective. In both of the late-maturing pigeonpea hybrids, variation for pollen production was also observed within the F_2 populations. Since at the time we recorded the data on segregation the information on the pollen load was not considered important, we could not study its relationship with the stability of fertility restoration. Tang et al. (2007) also observed partially fertile plants in a sorghum [*Sorghum bicolor* (L.) Moench] population that segregated for fertility-restoring alleles, and they reported that full pollen fertility in a genotype essentially results from the presence of all the major and minor genes simultaneously. They further postulated that the partial male fertility in the plants resulted from the absence of some fertility restoring alleles at minor-effect loci, which perhaps separated from the major-effect restoring genes during segregation and assortment processes. The present study shows that for the production of high-yielding

and stable pigeonpea hybrids, the selection for both of the dominant fertility-restoring genes is essential.

As we have observed here, fertility restoration in pigeonpea A_4 CMS lines is controlled by either one or two dominant genes in different male parents. Similarly, in other species, restoration of male fertility in their CMS systems involves only a small number of dominant genes: in pearl millet [*Pennisetum glaucum* (L.) R.Br.] from one to three (Yadav, 2005); in *Vicia faba*, one or two (Kaul, 1988); and in *Glycine max* (L). Merr., two (Bai and Gai, 2005). In most pigeonpea-growing countries, the traditional long-duration cultivars are grown in either pure or mixed stands on rainfed farms. In view of reduced landholdings linked to increasing population pressure and of the potential forces of climate change, the diversification of cropping systems has become essential. Pigeonpea, because of its special traits, such as drought tolerance and ability to recover from various biotic and abiotic stresses, is a favorite crop of farmers. Under these circumstances, breeding for high-yielding, early-maturing hybrids is likely to play an important role in the diversification of cropping systems. In this context, the results of this study related to the fertility restoration of hybrids will be valuable in the strategic planning of research and development programs for enhancing productivity and production of pigeonpea.

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