Indian J. Genet., **63**(3): 225-229 (2003)

Development of a cytoplasmic nuclear male-sterility system in pigeonpea using *C. scarabaeoides* (L.) Thouars.

K. B. Saxena and R. V. Kumar

International Crops Research Institute for the Semi-Arid Tropics, Patancheru 502 324

(Received: November 2002; Revised: August 2003; Accepted: August 2003)

Abstract

The phenomenon of heterosis has been successfully exploited for the genetic enhancement of yield in a number of cereal and horticultural crops. In pigeonpea (*Cajanus cajan* (L.) Millsp.) also, significant yield gains have been demonstrated through genetic male-sterility based hybrid technology. These hybrids, however, could not create the expected impact due to difficulties encountered in their large-scale seed production. To overcome this bottleneck, a cytoplasmic-nuclear male-sterility system was developed from a cross between *Cajanus scarabaeoides* (L.) Thouars, a wild relative of pigeonpea, and a short-duration pigeonpea cultivar. This paper besides summarizing the results of F_1 and backcross generations, reports the maintenance and fertility restoration systems of this newly developed cytoplasmic nuclear male-sterility source. The development of this technology is expected to help the seed industry in large-scale seed production of pigeonpea hybrids and their parents.

Key words: Pigeonpea, cytoplasmic-nuclear male-sterility, fertility restorer, maintainer

Introduction

Legumes, in general, are characterized with prominent cleistogamous flowers, which encourage high level of self-pollination. Pigeonpea (*Cajanus cajan* (L.) Millsp.), however, is an exception where natural out-crossing in the normal hermaphrodite flowers is common. It's large yellow and red coloured flowers attract a number of insects for foraging which affect cross-fertilization and its extent may range up to 70 % [1]. Pigeonpea breeders since long were on the look out for a stable male-sterility system which, in conjunction with natural out-crossing, could be used for commercial exploitation of hybrid vigour in this crop. To achieve this objective, stable genetic male-sterile (GMS) lines were identified from germplasm [2,3]. The hybrid breeding programmes using the improved GMS lines resulted in release of the world's first commercial pigeonpea hybrid ICPH 8 [4,5]. This hybrid, on average, demonstrated 25-30 % yield advantage over the best pure line variety in farmers' fields. Subsequently, five more pigeonpea hybrids were released by the Indian Council of Agriculture Research [6]. In spite of high yields and reasonable seed production costs, these hybrids could not become popular among seed growers because it necessitated manual roguing of fertile segregants from the female parent population [7]. This major hybrid seed production constraint could be overcome if an efficient cytoplasmic nuclear male-sterility (CMS) system is developed. In the past some efforts (8,9,10,11) have been made in this direction with moderate success towards developing a sound hybrid breeding technology. This paper reports the development of an efficient CMS system derived from a cross between *Cajanus scarabaeoides* (L.) Thouars, a wild relative of pigeonpea as female parent, and a short-duration pigeonpea cultivar.

Materials and methods

For developing CMS lines in pigeonpea, ICPW 89 - an Indian accession of *Cajanus scarabaeoides* (L.) Thouars (syn: *Atylosia scarabaeoides* (L.) Benth), a wild relative of pigeonpea and four pigeonpea lines ICPLs 87051, 87119, 88039 and ICP 8863 were selected. All the parental lines were sown on 18 July 1999 in Alfisols with recommended cultural practices. Flowering in ICPW 89 was profuse and continued from September to February and its four plants were selected randomly as female parent for hybridization. The emasculations on these plants were done and pollinations using the pigeonpea lines were performed soon after the emasculations. In each cross 50 pollinations were attempted. The inter-specific hybrid seeds were sown along with their parents on June 26, 2000 in Alfisols. At flowering each F_1 plant was examined for male-sterility / fertility. The F_1 progeny of cross ICPW 89 × ICPL 88039 was male-sterile and to produce BC_1F_1 seeds, it was crossed with recurrent parent ICPL 88039 and two other pigeonpea cultivars ICPL 88034 and ICPL 81. Subsequently, BC_2F_1, BC_3F_1, and BC_4F_1 seeds were also produced. The BC_2F_1 seeds were sown in Alfisol field while BC_1F_1, BC_3F_1 and BC_4F_1 generations were evaluated in a green house (Table 1). In Alfisol field the plantings were done on ridges, 75 cm apart with plant-to-plant spacing of

30 cm. In glasshouse, the materials were grown in 10" × 10" plastic pots filled with sterilized Alfisol mixture (4 parts Alfisol : 2 parts farm yard manure : 1 part sand).

To study fertility restoration of this CMS system in early generation, the male-sterile BC_2F_1 plants were crossed with 14 pigeonpea lines of diverse origin. The hybrid seeds and parents were sown in Alfisol field on June 23, 2002. The anthers of all the hybrid plants were examined physically for the presence / absence of pollen grains as well as by using the standard aceto-carmine staining test [12]. For this purpose 5-6 fully grown unopened floral buds were sampled randomly from each plant. Within each fertile F_1 progeny 10 plants were selected randomly at maturing for pod counts. To exclude the possibility of pod setting due to natural out-crossing, all the F_1 progenies were planted under an insect-proof nylon cage.

Results and discussion

Pod set in the inter-specific crosses ranged between 0 and 20 % resulting in only few seeds. In cross ICPW 89 × ICPL 87119 no pod set was observed. The germination of these F_1 seeds was also low and in two crosses only one plant germinated while in cross ICPW 89 × ICPL 88039 out of 30 seeds sown only three germinated. Hybridity of the F_1 plants was confirmed by comparing the morphological traits with their respective parents. All the F_1 plants were semi-creepers with vigourous canopy. The flower size, pod size and seed size were more or less intermediate between the two parents. In spite of small population the differences among F_1 progenies for male-sterility were apparent. The plants of crosses ICPW 89 × ICPL 87051 and ICPW 89 × ICP 8863 were fully male-fertile while the progeny of cross ICPW 89 × ICPL 88039 was completely male-sterile. In comparison to their normal fertile counterparts, anthers of the male-sterile plants were translucent, slightly less developed and completely devoid of pollen grains. To develop backcross populations the F_1 plants were crossed with ICPL 88039 (recurrent parent), ICPL 88034 and ICPL 81. In comparison to the initial inter-specific crosses, the pod set in backcrosses was better (30 to 38 %) suggesting greater cross compatibility of the parents. As expected, the plants in BC_1F_1 generation were erect and morphologically close to pigeonpea. All the plants in each backcross population were male-sterile (Table 1). Therefore, all the three pollinators were classified as maintainers of this newly developed CMS system. In the subsequent backcross (BC_2F_1 - BC_4F_1) generations also all the plants exhibited complete male-sterility, confirming the ability of these pollinators to maintain this CMS system. The expression of male-sterility was absolute both under open field conditions during rainy season and in glasshouse in winter, suggesting its stability of expression over such diverse environmental conditions. Detailed studies, however, are needed to confirm this observation. The three male-sterile populations were designated as CMS 88039A, CMS 88034A and CMS 81A.

In the alloplasmic male-sterility system where wild species are involved as cytoplasm donor, besides interaction between the two genomes various other cytoplasmic - genomic interactions also occur which lead to variable expression of male-sterility. Such negative interaction effects reduce gradually by each passing generation of selection and backcrossing. The present system, however, appears to be an ideal cytoplasmic-nuclear male-sterility system where a combination of nuclear and cytoplasmic genes produced

Table 1. Segregation for male-sterility in F_1 and backcross generations of crosses involving *C. Scarabaeoides* (ICPW 89) and three pigeonpea lines.

Cros	Date sown	Generation	Number of plants		
			Total	Fertile	Male-sterile
ICPW 89 × ICPL 87051	26 June 2000	F_1	1	1	0
ICPW 89 × ICP 8863	26 June 2000	F_1	1	1	0
ICPW 89 × ICPL 88039	26 June 2000	*F_1	3	0	3
*F_1 × ICPL 88039	12 Nov. 2000	BC_1F_1	140	0	140
*F_1 × ICPL 88034	12 Nov. 2000	BC_1F_1	0	0	60
*F_1 × ICPL 81	12 Nov. 2000	BC_1F_1	62	0	62
[(*F_1 × ICPL 88039) × ICPL 88039]	20 June 2001	BC_2F_1	246	0	246
[(*F1 × ICPL 88034) × ICPL 88034]	20 June 2001	BC_2F_1	35	0	35
[(*F_1 × ICPL 81) × ICPL 81]	20 June 2001	BC_2F_1	111	0	111
[(*F_1 × ICPL 88039) × ICPL 88039) × ICPL 88039]	06 Jan. 2002	BC_3F_1	230	0	230
[(*F_1 × ICPL 88034) × ICPL 88034) × ICPL 88034]	06 Jan. 2002	BC_3F_1	108	0	108
[(*F_1 × ICPL 81) × ICPL 81) × ICPL 81]	06 Jan. 2002	BC_3F_1	89	0	89
[(*F_1 × ICPL 88039) × ICPL 88039) × ICPL 88039) × ICPL 88039]	01 Sept. 2002	BC_4F_1	189	0	189
[(*F_1 × ICPL 88034) × ICPL 88034) × ICPL 88034) × ICPL 88034]	01 Sept. 2002	BC_4F_1	129	0	129
[(*F_1 × ICPL 81) × ICPL 81) × ICPL 81) × ICPL 81]	01 Sept. 2002	BC_4F_1	118	0	118

perfect male-sterile phenotypes. From such potential CMS source other promising CMS lines carrying desirable agronomic traits can easily be developed by using a standard backcross breeding programme.

Fertility restoration

The hybrid seed production technology based on cytoplasmic-nuclear male-sterility necessitates incorporation of fertility restorer gene(s) in the system. The cytoplasm donor species generally contains such fertility restoration genes. However, in the present case the attempts were made to identify such genes in the cultivated germplasm which would help in developing hybrids with acceptable agronomic and quality traits. According to Scoles and Evans [13] in majority of the plant species the fertility restoration is sporophytic in nature and all the pollen grains of plants either homozygous or heterozygous for fertility restoring gene(s) are functional. On the contrary, if the restoration is gametophytic in origin, the pollen grains are functional that carry only dominant form of restorer gene(s). In pigeonpea such information needs to be generated.

The plant population in 29 F_1s grown to study fertility restoration of this CMS system ranged considerably (Table 2). Therefore, in crosses where plant stand is low the results are interpreted with caution. CMS 88039A was crossed to all 14 male parents. Of these, in six crosses no pod set was observed and the examination of floral buds revealed that all the plants in each cross were male-sterile. In five crosses the fertility restoration ranged between 94 and 100% and in the remaining three crosses the fertility restoration was partial. In all the fertile and partial fertile progenies the pod set was good and in certain hybrids it matched with that of control ICPL 87119 (Table 2). The information generated from crosses with CMS 88034A the parents FRC 1, 2, and 3 were classified as full restorers and FRC 6 and 7 as partial restorers. The remaining three F_1 progenies were male-sterile with no pod set. Five hybrids involving CMS 81A male-sterile were evaluated and only one restored the male-fertility. Considering the overall data, a close similarity in fertility restoring ability of the pollen parents across the three CMS lines was observed. Six pollen parents (FRS 9 to 14) maintained the male-sterility in every combination. From this group, based on important agronomic characters of the maintainers, one or two crosses will be selected for further backcrossing to diversify the genetic base of the CMS system. This will help in developing genetically diverse pigeonpea hybrids for different agro-ecological conditions and cropping systems. All the hybrids involving pollinators FRS 1,2,3,4 and 5 exhibited high levels of fertility restoration. These parents need to be preserved for future use and in breeding high yielding restorer lines. Six hybrids involving parents FRS 6, 7 and 8 were found segregating for male-sterility and fertility. Such pollinators cannot be used directly in the hybrid breeding programme and to improve their fertility restoration ability, a breeding programme for genetic purification for fertility restoration will be necessary.

A close perusal of fresh matured anthers revealed significant variation within F_1 progenies for the quantity of pollen produced and only in a few plants the anthers had pollen load similar to that of control ICPL 87119. Further examination under microscope also revealed that in comparison to control, the pollen grains of the hybrid plants were relatively smaller in size. Due to

Table 2. Frequency (%) of fertile plants and average pod number in F_1 hybrids between three CMS lines and 14 locally adapted cultivars of pigeonpea

Pollen parent	CMS 88039A		CMS 88034A		CMS 81A	
	Fertile plants (%)	Pods/ plant	Fertile plants (%)	Pods/ plant	Fertile plants (%)	Pods/ plant
FRS 1	99 (96)	333.3±23.45	95 (21)	480.0±22.04	100 (8)	418.3±14.65
FRS 2	94 (49)	356.0±21.81	100 (4)	550.0±13.68	-	-
FRS 3	100 (29)	371.0±22.64	100 (4)	585.0±7.83	-	-
FRS 4	100 (45)	421.3±17.89	-	-	- -	-
FRS 5	100 (53)	677.3±30.76	-	-	-	-
FRS 6	81 (43)	285.3±14.50	86 (36)	377.5±23.90	-	-
FRS 7	77 (44)	275.0±12.47	56 (16)	210.0±4.10	50 (22)	301.0±8.89
FRS 8	68 (34)	297.1±7.12	-	-	-	-
FRS 9	0 (69)	0	-	-	0 (11)	0
FRS 10	0 (44)	0	0 (9)	0	0 (5)	0
FRS 11	0 (14)	0	0 (24)	0	0 (17)	0
FRS 12	0 (14)	0	-	-	0 (21)	0
FRS 13	0 (13)	0	0 (3)	0	0 (3)	0
FRS 14	0 (21)	0	-	-	-	-
ICPL 87119 (control)		487.4±23.02				

( ) Number of plants

technical limitations the quantification of this variation was not possible.

Five F_1 hybrids exhibiting high level of male-fertility restoration and reasonable plant population were selected to study the intra-population variation for pollen viability along with cultivar ICPL 87119 as control. The mean pollen viability in the control was over 95% while within the F_1 hybrid progenies a large variation for this trait was observed (Table 3). Out of 272 plants examined in five hybrids, only 33 exhibited pollen viability similar to that of control. In cross CMS 88039A × FRS 3, out of 29 plants studied 25 exhibited more than 80% pollen viability. More or less similar results were obtained in crosses CMS 88039A × FRS 4 and CMS 88039A × FRS 5. These observations showed that the fertility restoration within hybrid progenies was not uniform and up to the mark and it may be due to differential inter-genomic or cytoplasmic-genomic interactions. Such interactions usually involve complex genetic phenomenon like complementation, inhibition, epistasis, accumulation, etc. which render the male-fertility restoration control highly subtle and fragile. In addition, some times environment also plays an important role in the expression of sterility/fertility [14]. According to Abdalla and Hermsen [15] the polymorphism, arising due to differential genes also results in inconsistent male-sterility / fertility expressions and produce abnormal pollens. In the present case the hybrid combinations were developed using early generation (BC_2F_1) male-sterile plants. In this material about 12.5 % C. scarabaeoides genome still remained and it means that the genome of recurrent parent placed in the cytoplasm of the female line has deficit genes by about the same proportion and the interaction of this residual genome with that of pigeonpea may be responsible for restricting the full restoration of male-fertility of the substitution lines. In the present study some pollinators such as FRS 1, 2 and 3 have shown promise for male-fertility restoration and it is presumed that further improvement of CMS lines through backcrossing and selection may enhance the expression of male-fertility restoration within hybrid progenies.

Table 3. Variation for pollen stainability (%) within five F_1 hybrid populations involving CMS 88039A

Pollen stained (%)	ICPL 87119 (control)	Crosses of CMS 88039A with				
		FRS 1	FRS 2	FRS 3	FRS 4	FRS 5
0 (sterile)	0	1	3	0	0	0
01 - 09	0	0	0	0	0	0
10 - 19	0	1	0	0	0	0
20 - 29	0	1	2	0	0	2
30 - 39	0	7	2	0	0	1
40 - 49	0	18	6	0	1	1
50 - 59	0	10	4	1	4	1
60 - 69	0	17	4	1	5	6
70 - 79	0	5	5	2	12	8
80 - 89	0	25	17	14	12	16
90 - 94	0	7	3	2	4	8
> 95	23	4	3	9	7	10
Total plants	23.	96	49	29	45	53

General discussion

In spite of sincere breeding efforts in various research institutions, the global mean productivity of pigeonpea has remained unchanged for decades at around 0.6 - 0.7 t ha^{-1}. The development of new pure line varieties, particularly those with shorter maturity, has helped in extending pigeonpea cultivation in new niches, but it did not help increasing the productivity of this crop. The hybrid pigeonpea breeding technology, developed by exploiting its limited natural out-crossing and genetic male-sterility, offered an opportunity for the genetic enhancement of yield in this legume. Although this technology ably demonstrated 25-30% yield gains in farmers' fields, it failed to take-off commercially due to some inherent practical problems associated with any GMS-based hybrid seed production system. The results of present study leads to an optimism of reviving hybrid pigeonpea breeding technology that would overcome the seed production constraints encountered earlier with the GMS system. The CMS lines developed under this project appear agronomically productive with no major deficiencies. Further, it has also been demonstrated that this male-sterility trait can easily be transferred to other genotypes of different phenologies. The identification of male-fertility restoration of this CMS system offers scope for producing heterotic hybrids suited to different agro-ecological and cropping system niches. To achieve this objective, a follow-up breeding programme, however, is necessary to develop new genetically diverse hybrid parents with high combining ability.

Among legumes, CMS systems have been developed in alfalfa (*Medicago sativa*) [16] and broad bean (*Vicia faba*) [17] but the commercial exploitation of hybrid vigour in these crops was not feasible due to insufficient natural pollen transfer from male to female parents for facilitating large scale hybrid seed production. The extensive research conducted on GMS-based pigeonpea hybrid technology, however, clearly demonstrated that this crop has sufficient level of commercially exploitable hybrid vigour and the extent of natural out-crossing is not a limitation in producing large quantities of hybrid seed. The constraints related to the maintenance and use of female parent experienced earlier will soon be overcome by using CMS lines reported herein and soon the farmers' would be able to reap the benefits of this technology.

References

1. **Saxena K. B., Singh L. and Gupta M. D.** 1990. Variation for natural out-crossing in pigeonpea. Euphytica, **46**: 143-148.

2. **Reddy B. V. S., Reddy L. J. and Murthi A. N.** 1977. Reproductive variants in *Cajanus cajan* (L.) Millsp. Trop. Grain Leg. Bull., **7**: 11.

3. **Saxena K. B., Wallis E. S. and Byth D. E.** 1983. A new gene for male-sterility in pigeonpeas. Heredity, 51: 419-421.

4. **Saxena K. B., Chauhan Y. S., Kumar R. V., Ariyanayagam R. P., Johansen C. and Asthana A. N.** 1997. Heterosis breeding for higher yield in pigeonpea. *In* : A. N. Asthana and Masood Ali (eds.), Recent Advances in Pulses Research. Indian Institute of Pulses Research, Kanpur, India. p. 109-125.

5. **Saxena K. B., Gupta S. C., Kumar R. V., Reddy L. J., Singh L. and Faris D. G.** 1989. ICPH 8 - the first promising short-duration pigeonpea hybrid. International Pigeonpea Newsletter, **9**: 8-10.

6. **Vishwa Dhar, Singh N. B., Wanjari K. B., Singh F. and Majumder N. D.** 2002. Pigeonpea nucleus and breeder seed production. Indian Institute of Pulses Research, Kanpur, India. pp 20.

7. **Niranjan S. K. D. F. F., Bantilan, M. C. S., Joshi P. K. and Saxena K. B.** 1998. Evaluating hybrid technology of pigeonpea. *In*: Bantilan M.C.S. and Joshi P.K. (eds.), Assessing Joint Research Impacts: Proc. Intl. Workshop on Joint Impact Assessment of NARS/ICRISAT Technologies for the Semi-arid Tropics, 2-4 Dec 1996. ICRISAT, Patancheru, AP 502 324, India. p. 231-240.

8. **Reddy L. J. and Faris D. G.** 1981. A cytoplasmic-genic male-sterile line in pigeonpea. International Pigeonpea Newsletter, **1**: 16-17.

9. **Ariyanayagam R. P., Nageshwar Rao A. and Zaveri P. P.** 1995. Cytoplasmic-genic male-sterility in interspecific matings of *Cajanus*. Crop Sci., **35**: 981-985.

10. **Tikka S. B. S., Parmar L. D. and Chauhan R. M.** 1997. First record of cytoplasmic-genic male-sterility system in pigeonpea (*Cajanus cajan* (L.) Millsp.) through wide hybridization. GAU Res. J., **22**: 160-162.

11. **Wanjari K. B., Patil A. N., Manapure P., Manjayya J. G. and Manish P.** 2001. Cytoplasmic male-sterility in pigeonpea with cytoplasm from *Cajanus volubilis*. Ann. Plant Physiology, **13**: 170-174.

12. **Kaul C. L. and Singh S. P.** 1969. Validity of stain tests in determining pollen viability of some papilionaceous plants. Indian J. Agric. Sci., **39**: 1050-1055.

13. **Scoles G. J. and Evans L. E.** 1979. The genetics of fertility restoration in cytoplasmic male-sterile rye. Can J. Genet Cytol., **21**: 417-422.

14. **Kaul M. L. H.** 1988. Male sterility in higher plants. Springler - Verlag. New York pp. 1005.

15. **Abdalla M. M. F and Hermsen J. G. T.** 1972. Plasmons and male-sterility types in *Solanum verrucosum* and its interspecific hybrid derivations. Euphytica, **21**: 209-220.

16. **Bradner N. R. and Childers W. R.** 1968. Cytoplasmic male-sterility in alfalfa. Can. J. Plant Sci., **48**: 111-112.

17. **Bond D. A., Fyfe J. L. and Toynbee-Clarke G.** 1966. Male-sterility in field beans IV. Use of cytoplasmic male-sterility in the productin of F1 hybrids, and their performance in trials. J. Agric. Sci., **66**: 369-377.