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A cytoplasmic-nuclear male-sterility system derived from a cross between *Cajanus cajanifolius* and *Cajanus cajan*

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Summary

Cytoplasmic-nuclear male-sterility is an important biological tool, which has been used by plant breeders to increase yields in cross-pollinated cereals and vegetables by commercial exploitation of the phenomenon of hybrid vigor. In legumes, no such example exists due to the absence of an economic way of mass pollen transfer from male to female parent. Pigeonpea [*Cajanus cajan* (L.) Millsp.], however, is a different legume where a moderate level of insect-aided natural out-crossing (25–70%) exists and it can be used to produce commercial hybrid cultivars, if an efficient and stable cytoplasmic-nuclear male-sterility (CMS) system is available. This paper reports the development of a stable CMS system (ICP 2039A), derived from an inter-specific hybrid of *Cajanus cajanifolius*, a wild relative of pigeonpea, with a cultivar ICP 11501. Using this genetic material, designated as the A_4 cytoplasm, a number of fertility restorers and maintainers have been developed. The best short-duration experimental pigeonpea hybrid ICPH 2470 produced 3205 kg ha^{-1} grain yield in 125 days, exhibiting 77.5% advantage over the control cultivar UPAS 120. At present, all the important biological systems necessary for a successful commercial hybrid breeding program are available in pigeonpea and the package of this technology has been adopted by private seed sector in India for the production and marketing of hybrid varieties.

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is an important food legume crop grown on over four million hectares globally, mainly in tropics and sub-tropics under subsistence agriculture by resource-poor farmers. The importance of pigeonpea in rainfed agriculture is on the increase (Ryan, 1997) due to steady changes in various social and climatic factors. The shortage and unpredictability of rainfall, long and intermittent dry spells, reduction in the size of farm holdings, and inability of the farmers to purchase inputs are some of the primary reasons for choosing pigeonpea over other rainy season field crops. Research efforts for enhancing the productivity of pigeonpea through traditional pure line breeding have not been successful and for the past five decades it has remained steady at around 700 kg ha^{-1} (Saxena et al., 2005).

In general, legumes are highly self-pollinating. Pigeonpea is different from other legumes in being often classified as an 'often cross-pollinated crop' with 25–70% natural out-crossing reported for different locations (Saxena et al., 1990). The breeders, however, by deploying various hybridization and selection schemes have always considered pigeonpea a self-pollinating crop. Khan (1973), Onim (1981), and Byth et al. (1981) recommended various population improvement schemes in pigeonpea using natural out-crossing but so far no high-yielding population has been developed and it has remained as an academic exercise only.

In 1974, pigeonpea breeders at ICRISAT planned to use the natural out-crossing for developing high yielding hybrids and an extensive search was made for an effective male-sterility system within the germplasm collection. This exercise resulted in the selection of a stable genetic male-sterility (GMS) system (Reddy

et al., 1978). This material was used to develop a GMS-based hybrid breeding technology and after 6 years of extensive multilocation testing, the world's first food legume hybrid ICPH 8 was released for cultivation in India (Saxena et al., 1992). This hybrid had a 25–30% yield advantage over the control in farmers' fields. This demonstrated that heterosis in pigeonpea can be commercially exploited. However, due to the genetic nature of the male-sterility system large-scale production of quality seed of the female parents and of hybrids was not feasible which resulted in poor adoption of the hybrid. To overcome this constraint, it was essential to develop a cytoplasmic-nuclear male-sterility (CMS) system in pigeonpea. In the past few years, three such CMS systems have been reported in pigeonpea. These are (i) A_1 cytoplasm, derived from *C. sericeus* (Ariyanayagam et al., 1995; Saxena et al., 1996); (ii) A_2 cytoplasm, derived from *C. scarabaeoides* (Tikka et al., 1997; Saxena & Kumar, 2003); and (iii) A_3 cytoplasm, derived from *C. volubilis* (Wanjari et al., 2001). The instability in the expression of male-sterility and their fertility restoration, however, has limited their use in large-scale practical hybrid breeding programs (Saxena et al., 2005). This paper reports the development of an efficient CMS system ICP 2039A that was derived from a hybrid involving *Cajanus cajanifolius*, the genetically closest wild relative of pigeonpea, and a cultivated line ICP 11501.

Materials and methods

ICPW 29, an accession of *C. cajanifolius* (Haines) van der Maesen *comb. nov.* ($2n = 22$), a wild relative of pigeonpea, was crossed as the female parent with a short-duration cultivar ICP 11501 ($2n = 22$) during the 1999 rainy season. According to De (1974), *C. cajanifolius* resembles the cultivated type in numerous morphological traits and differs only by a single gene. The inter-specific F_1 hybrid seeds were sown in field in the 2000 rainy season. Mature anthers of the F_1 plants were examined for the presence/absence of pollen grains and their fertility/sterility was studied under microscope using 2% aceto-carmine solution. One F_1 plant with the lowest pollen fertility (40%) was selected for backcrossing with ICP 11501. Subsequently, six backcrosses (BC_2–BC_7F_1) were made to substitute the genome of *C. cajanifolius* with that of the cultivated type. Seeds of all backcross generations except BC_6F_1 and BC_7F_1 were grown in a glasshouse in 25 cm × 25 cm plastic pots filled with sterilized Alfisol mixture (4 parts Alfisol:2 parts farm yard manure:1 part sand).

The male-sterile BC_6F_1 population of this cross-combination was designated as ICP 2039A and its maintainer (ICP 11501) as ICP 2039B. For large-scale seed multiplication, these lines were planted in isolation on 16 June 2004 in five sets, each consisting of 1:4 [male (ICP 2039B):female (ICP 2039A)] rows. The row-to-row and plant-to-plant spacings were kept at 75 and 25 cm, respectively. For morphological characterization of the CMS source, five plants were randomly selected in each of the five sets. The data were recorded for each plant for days to first flower, plant height (cm), number of primary branches per plant, pods per plant, pod length (cm), seeds per pod, and 100 seed mass (g). The data from all the five sets were pooled to calculate the mean and standard errors for each trait. The anthers of all 1133 plants of ICP 2039A were examined for the presence of pollen grains and their viability. On January 2, 2005, a total of 738 BC_7F_1 plants were again planted to confirm the expression male-sterility.

To study the fertility restoration and maintenance of ICP 2039A, the male-sterile BC_5F_1 plants were crossed with 86 early maturing pigeonpea lines and the resultant F_1 progenies were grown in subsequent rainy season along with control UPAS 120. Of these, eight cross combinations, where the number of seeds was high, were evaluated for their agronomic features in a trial along with UPAS 120 as check. A basal dose of diammonium phosphate was applied at 100 kg ha^{-1} to provide 18 kg N and 46 kg P. Each entry was sown in a 4-row plot with two replications. The sowing was done on July 1, 2004 on ridges, 75 cm apart with plant-to-plant spacing of 20 cm. The trial was irrigated four times and three hand weedings were done to control weeds. In each plot, data on days to 75% maturity and grain yield were recorded on plot basis. The single plant observations were recorded on five randomly selected competitive plants as described earlier.

Results and discussion

Development of CMS line ICP 2039A

A total of 150 pollinations were made on ICPW 29 using fresh pollen from ICP 11501. The success rate was low and only 9 pods were set that produced 16 hybrid seeds, of which 12 germinated. The anthers of these plants were fully grown, light yellow in color and contained small amounts of pollen. The aceto-carmine

Table 1. Male-sterility in F_1 of cross ICPW 29 (*C. cajanifolius*) × ICP 11501 (*C. cajan*) and backcross generations used in the development of CMS line ICP 2039A

Generation	Year	Location	Number of plants		Male-sterility (%)
			Total	Male-sterile	
F_1	2000	Field	12	12[a]	–
BC_1F_1	2001	Glasshouse	8	8	100
BC_2F_1	2002	Glasshouse	5	4+1[a]	80
BC_3F_1	2002	Glasshouse	165	165	100
BC_4F_1	2003	Glasshouse	7	7	100
BC_5F_1	2003	Glasshouse	67	67	100
BC_6F_1	2004	Field	1133	1133	100
BC_7F_1	2005	Field	738	738	100

[a]Partial male-fertile.

test revealed partial pollen fertility in each plant and it ranged from 40 to 80%. All eight BC_1F_1, plants were completely male-sterile (Table 1) with no trace of pollen grains. In BC_2F_1, out of five plants grown four were male-sterile. In the subsequent backcross generations (BC_3F_1–BC_7F_1), all plants were male-sterile. In BC_2F_1 and BC_4F_1 water-logging in pots caused severe damage to plants.

Morphological description of ICP 2039A

Overall, there were no marked differences between the male-sterile line (ICP 2039A) and its maintainer (ICP 2039B) in plant morphology and other agronomically important characteristics. The plants of this male-sterile source were determinate and compact. Stems were green and flowers yellow with red veins. Anthers were fully grown with light yellow in color and no pollen grains. The plants of ICP 2039A grow up to 126.2 ± 1.0 cm in height and flower in 66.8 ± 0.2 days after planting (Table 2). The green colored pods with dense purple streaks are 5.74 ± 0.05 cm long and, on average, they produce 3.32 ± 0.07 seeds. The seeds of ICP 2039A are round and brown in color with 100-seed mass of 10.3 ± 0.15 g.

Table 2. Some agronomic features of ICP 2039A and its maintainer line ICP 2039B recorded at Patancheru, 2004 rainy season

Trait	ICP 2039A	ICP 2039B
Days to first flower	66.8 ± 0.2	67.1 ± 0.2
Plant height (cm)	126.2 ± 1.0	126.1 ± 2.1
Number of primary branches	15.3 ± 0.34	14.3 ± 0.95
Pod length (cm)	5.74 ± 0.051	5.97 ± 0.085
Seeds pod^{-1}	3.32 ± 0.067	3.50 ± 0.130
100-seed mass (g)	10.3 ± 0.15	9.8 ± 0.05
Number of pods plant^{-1}	137.1 ± 4.06	125.3 ± 3.01

In comparison to ICP 2039B, the A-line took about 2 additional weeks to mature and produced more pods (137.1 ± 4.06). The similar results were also recorded in a seed production study of genetic male-sterile and fertile segregants (Saxena et al., 2005). These observations are in contrast to most crops where CMS systems have been developed, and its reasons are primarily physiological. Sheldrake (1979) and Sheldrake and Narayanan (1979) conducted a series of experiments to understand the process of pod setting and pod development in pigeonpea and concluded that the pigeonpea crop is grossly different from other food legumes as far as its source–sink relationship is concerned. They attributed it to the woody and intrinsically perennial nature of pigeonpea.

The pigeonpea plant produces a large amount of photosynthates but less than 20% of it is consumed in producing the seeds, and the remaining dry matter is conserved within the plant to support its life system under unfavorable conditions (Chauhan et al., 1992). Sheldrake (1979) and Sheldrake and Narayanan (1979) also demonstrated that in pigeonpea, the grain yield was not limited by nutrient supply but it is a direct consequence of the number of pods set on a plant. Therefore, the pod setting on an individual plant stops when its food reserves fall below a threshold. Such threshold levels are not permanent and may vary from one cultivar to other and within a season depending on the prevailing macro/micro environmental conditions.

The male-fertile maintainer (B) plant, like other pigeonpea cultivars, flowers profusely but only 5–10% of its flowers set pods. Most pods in the fertile plant develop from the first flush of flowers and almost attain its potential pod set. The majority of the late-emerging flowers drop even after their fertilization. These events, as mentioned earlier, are directly linked to the source–sink relationships. On the other hand, in the male-sterile plant, the initial pod set is low because it is entirely dependent on the number of insect pollinators. At this point of time, the number of pods set on the male-sterile plant is well below its threshold, which permits the formation of additional pods with subsequent pollinations. This process of insect-dependent pod setting on the male-sterile plant is slow and continues for a relatively longer period to reach its threshold level, resulting in some delay of maturity. The slow pod setting perhaps also enhances the threshold capacity of the

male-sterile plant that allows it to hold relatively more pods than its fertile counterpart. However, this needs confirmation through some specific physiological studies with A and B—lines of a CMS system.

Fertility restoration

Out of 86 F_1 hybrid progenies evaluated for fertility restoration, 14 (16.28%) fully restored male-fertility; 17 (19.77%) were found to maintain male-sterility; and the remaining 55 (63.95%) segregated for male-sterility and fertility. Among the fertile hybrid combinations, the mean pollen fertility (Table 3) ranged from 89.89 ± 3.84 to 99.13 ± 0.3%. However, the perusal of intra hybrid data reveals some variation among plants for fertility restoration. In cross combinations involving ICPL 92044 (mean 96.49 ± 1.66%), pollen fertility among plants ranged from 83.33 to 99.66%. It may either be due to the presence of some degree of variability within the pollen parent in its ability to bring about full restoration of male-fertility in the F_1 hybrids. Such intra-accession variation for fertility restoration can easily be minimized or eliminated by making plant-to-plant crosses between male-sterile and fertility restoring line, and selecting the best fertility restoring male plants. Such single plants can be used to produce nucleus seed of restorer lines. The other reason for this event could be the presence of differential intergenomic or cytoplasmic-genomic interactions. Such interactions usually involve complex genetic functions like complementation, inhibition, epistasis, accumulation, etc. which render the male-fertility restoration control highly subtle and fragile (Kaul, 1988). According to Abdalla and Hermsen (1972), the polymorphism, arising due to differential genes, also results in inconsistent male-sterility/fertility expressions. Additionally, environment may also play an important role in the expression of male-sterility/ fertility.

Table 3. Male-sterility maintainers and male-fertility restorers identified in cross combinations involving ICP 2039A and fertile inbred lines at Patancheru, 2004 rainy season

Male-sterility maintainers		Male-fertility restorers	
Genotype	Pollen fertility (%)	Genotype	Pollen fertility (%)
ICPL 91030	0.83 ± 0.24	ICPL 20139	89.89 ± 3.84
ICPL 118-2	1.33 ± 0.36	ICPL 93105	92.33 ± 2.85
ICPL 20155	1.53 ± 0.29	ICPL 99047	94.88 ± 1.04
ICPL 20157	1.62 ± 0.43	ICPL 93101	96.20 ± 1.57
ICPL 85010	1.83 ± 0.74	ICPL 20201	96.26 ± 0.94
ICPL 20159	2.08 ± 0.73	ICPL 92044	96.49 ± 1.66
ICPL 20176	3.32 ± 0.68	ICPL 20203	98.06 ± 0.52
ICPL 95039	3.85 ± 1.20	ICPL 20202	98.23 ± 0.32
ICPL 92016	3.91 ± 0.61	ICPL 92043	98.63 ± 0.48
ICPL 98009	4.25 ± 1.48	ICP 8741	99.13 ± 0.30
ICPL 86005	4.49 ± 0.64	ICP 10934	91.09 ± 3.33
ICPL 20156	4.86 ± 1.01	ICP 8094	93.65 ± 2.09
ICPL 265-1	6.16 ± 3.64	ICP 11892	95.89 ± 1.44
ICPL 98012	8.41 ± 1.42	ICP 9880	96.13 ± 0.83
ICPL 86022	8.61 ± 1.03	UPAS 120	97.1 ± 0.96
ICP 14712	2.76 ± 0.56	(check)	
ICP 15600	7.25 ± 1.14		

In four hybrids, cross combinations involving ICPL 20203, 20202, 92043, and ICP 8741, the pollen fertility compared well with that of control UPAS 120 (Table 3) and the male parental lines of these F_1s can be recommended as restorers for direct use in hybrid pigeonpea breeding programs. The special feature of the fertility restoration of this group of material is that the quantity of pollen produced in each fertile F_1 plant matched well with that of control. It is considered a positive indication of high quality of fertility restoration that will assist in setting a high number of pods on the hybrid plants. In contrast, the experimental hybrids derived from the A_1 and A_2 cytoplasms are shy bearers and produce less pollen (Saxena and Kumar, 2003). It appears that in general the cytoplasm of *C. cajanifolius*, which is considered the progenitor of the cultivated pigeonpea (van der Maesen, 1980), interacts more or less normally with the genome of the cultivated type to produce high pollen bearing hybrid plants.

Among the 17 F_1 hybrid progenies that maintained male-sterility (Table 3), the mean pollen fertility ranged from 0.83 ± 0.24 to 8.61 ± 1.03%. In 13 hybrid combinations, the mean pollen fertility was less than 5% and these male-sterile hybrid combinations could be used for backcrossing to develop new A-lines. In the remaining 55 F_1 hybrids, large variation was observed for mean pollen fertility (13.90±3.83 to 81.33±3.48%). In these hybrids, selection can be exercised to breed either fertility restorers or male-sterility maintainers. In fact, it will be difficult for breeders to use all segregating F_1 hybrid progenies in breeding. They should carefully study the important agronomic traits of parental lines and based on the need of genetic diversity in their programs, choose the pollinators of hybrid combinations either for fertility restoration or male-sterility maintenance.

Table 4. Performance of eight early maturing pigeonpea hybrids at Patancheru, 2004 rainy season

Entry	Days to mature	Plant height (cm)	Seeds pod^{-1}	100-seed mass (g)	Pods plant^{-1}	Plant stand	Fertility restoration (%)	Grain yield		Heterosis over check (%)
								(g plot^{-1})	(kg ha^{-1})	
ICPH 2470	125	190	3.8	8.9	232	38	100	1.73	3205	77.5
ICPH 2438	122	160	3.8	9.0	181	36	100	1.30	2404	33.1
ICPH 2429	128	180	3.8	9.4	136	36	100	1.27	2351	30.2
ICPH 2431	122	169	3.4	8.3	160	34	100	1.19	2195	21.5
ICPH 2472	135	190	3.9	9.1	194	32	100	1.16	2150	19.0
ICPH 2433	125	162	3.2	8.5	144	31	100	1.14	2118	17.3
ICPH 2436	128	155	3.2	10.5	142	34	100	1.12	2067	14.4
ICPH 2457	122	170	3.5	9.3	126	35	100	1.01	1876	3.9
UPAS 120 (check)	128	160	3.4	9.1	114	50	–	0.98	1806	–
S.E.M.	±3.3	±10.2	±0.14	±0.23	±37.6	±2.6	–	±0.093	±171.5	–
CV (%)	3.6	8.5	5.63	3.48	33.4	10.3	–	10.8	10.8	–

Evaluation of experimental hybrids

The differences among test entries were highly significant for various traits and all hybrids produced greater seed yield than control UPAS 120 (1806 kg ha^{-1}). Fertility restoration in all evaluated hybrids was perfect (Table 4). The best one, ICPH 2470, matured in 125 days and produced 3205 kg ha^{-1} seed yield, 77.5% yield advantage over the check. This hybrid also had acceptable seed size and produced the highest number of pods. The estimates of productivity per unit of time indicated that ICPH 2470 produced 25.6 kg ha^{-1} day^{-1} grain as compared to 14.1 kg ha^{-1} day^{-1} in the control. From this trial, three hybrids ICPH 2470, ICPH 2438, and ICPH 2429 have been selected for further testing.

Conclusions

So far, 63 pure line cultivars of pigeonpea have been released for cultivation in India (Singh et al., 2005), but the plateuing of low yield levels for the past five decades is a serious concern. The development of the CMS-based hybrid pigeonpea technology appears to be the logical way to achieve a significant breakthrough in the yield potential of the crop (Saxena et al., 2005). The new source of CMS, reported herein and designated as the A_4 cytoplasm, was developed using *C. cajanifolius*, a wild relative of pigeonpea. This wild species is reported to be genetically closest to the cultivated type and differs only by a single gene (De, 1974). The male-sterile plants in this material show no morphological deformity and produce plenty of pollen in hybrid combinations with restorers. The frequency of fertility restorers is good and the evaluation of limited number of hybrid combinations (Saxena & Kumar, 2005) presents an optimistic view of the hybrid breeding technology in enhancing the yield levels in pigeonpea.

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