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RESEARCH ARTICLE

Development of a *Cajanus cajanifolius*-based CMS Hybrid Technology in Pigeonpea

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Abstract

Pigeonpea is an important food legume of the semi-arid tropics grown mainly under subsistence agriculture. As a first step to address the five decades-long yield stagnation, a proof-of-concept for a commercially viable CMS-based hybrid technology with high standard heterosis and fertility restoration has been demonstrated. Six generations of backcrossing and selection for male sterility, and agronomic superiority using a *Cajanus cajanifolius*- (A_4 cytoplasm) based CMS donor (ICPA 2039) and a medium-maturity group recurrent parent ICPL 20176 (ICPB 2043) which resulted in a stable male sterile line (ICPA 2043) with near-perfect male sterility and superior agronomic traits. Following backcrossing and selections, the A-line (ICPA 2043) appeared to be morphologically similar in terms of various qualitative and quantitative traits compared to the B-line (ICPB 2043). However, differences between ICPA 2043 and ICPB 2043 were observed for days to maturity, 100-seed weight, pods plant^{-1}, and seed weight plant^{-1}. These differences may not be genetic but physiological. The best heterotic restorer line selected in a cross (ICPH 2671) combination restored a mean of 96.49% male fertility in 36 environments (12 locations, 3 years). Present investigation did not reveal significant G×E interaction for fertility restoration, indicating the possibility of obtaining high and stable grain yields in the pigeonpea growing areas of South and Central India.

Key words : *Cajanus cajan*, CMS, fertility restoration, G×E, hybrid, pigeonpea

Introduction

To feed the burgeoning human population, global food grain production needs to take a quantum jump primarily aided by increased productivity. Hybrids in cereals, oilseeds, and vegetables have shown great promise in breaking the yield barrier. Pigeonpea (*Cajanus cajan* (L.) Millisp.) is a novel addition to the group of hybrids in food legumes. It is an important subsistence crop of the semi-arid tropics. Pigeonpea is grown under different cropping patterns and imparts sustainability to various production systems (Khoury et al. 2015). Globally, pigeonpea is cultivated in more than 20 countries on 5.40 million hectares with an annual production of 4.48 million tons (FAOSTAT 2018). Grown under resource poor conditions between latitudes of 40^0 S and 40^0 N, it is one of the principal grain legumes of Asia, Africa, and Latin Americas. It has good phenotypic plasticity such as drought tolerance, the ability to recover from the losses caused by various stresses, along with its use as fodder and fuel wood (Lose et al. 2003; Odeny et al. 2007). Besides its main use as protein-rich (22-24%) dal (dehulled split peas), its immature green seeds and pods are consumed as a vegetable (Kenya, Tanzania, Malawi) that can also be processed for canning and freezing (China, Philippines, and the Caribbean). Pigeonpea is a valuable source of protein to the largely vegetarian population of India, and is part of the daily cuisine (Saxena et al. 2010). In Africa it provides nutritional security to millions of poor, and is also consumed with maize (Mula and Saxena 2010).

In the past half century, the global productivity of pigeonpea has remained discouragingly low at around 650-850 kg ha^{-1}. This is primarily due to the non-availability of high-yielding cultivars and susceptibility to major diseases such as *Fusarium* wilt and sterility mosaic. India is the world's largest producer and consumer of pigeonpea, accounting for 3.88 m ha area followed by Myanmar (0.66 m ha), Tanzania (0.26 m ha), Malawi (0.24 m ha), Haiti (0.13 m ha), and Kenya (0.11 m ha) (FAOSTAT 2018). In India, the gap between demand

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and production levels have been ever increasing leading to a steep rise in the pigeonpea prices and a significant decline in per capita availability of vegetable protein through pulses in general and pigeonpea in particular (Saxena et al. 2006). To address the low productivity in pigeonpea, scientists at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) envisaged the idea of producing hybrids since ample heterosis was reported in past studies (Saxena and Sharma 1990; Solomon et al. 1957). Later, Sharma and Dwivedi (1995) postulated the role of additive and additive × additive inter-allelic and inter-genomic interactions, besides over-dominance and dominance gene actions in the manifestation of heterosis.

In the pursuit of genetic male sterility (GMS)-based hybrid development, Reddy et al. (1978), were the first to report a translucent anther-type male sterile line carrying the *ms1* gene. Later, Saxena et al. (1983) reported another source of genetic male sterility controlled by a different non-allelic gene *ms2* responsible for shriveled, arrowhead-shaped, non-dehiscent, brown anthers. These sources were used to generate the world's first GMS-based hybrid, ICPH 8. In multi-location trials conducted in different agro-ecological zones of India, ICPH 8 gave 41% yield superiority over the commercial cultivar UPAS 120 (Saxena et al. 1992). The GMS-based hybrids, though high yielding could not become popular with farmers due to inherent problems like the need for rouging out fertile segregants in the seed production blocks. The first attempt to develop a stable CMS system was by Reddy and Faris (1981), wherein a wild-type *Cajanus scarabaeoides* was crossed with cultivated pigeonpea. Subsequently Ariyanayagam et al. (1995) crossed another wild species, *Cajanus sericeus* with cultivated pigeonpea. Both attempts did not yield an efficient male sterility system. Consequently, efforts were made to breed for CMS using several inter- and intra-specific donors. These are designated as (i) A_1 cytoplasm, derived from *C. sericeus* (Saxena et al. 1996); (ii) A_2 cytoplasm, derived from *C. scarabaeoides* (and Kumar 2003; Tikka et al. 1997); (iii) A_3 cytoplasm, derived from *C. volubilis* (Wanjari et al. 2001); (iv) A_4 cytoplasm derived from *C. cajanifolius* (Saxena et al. 2005); and (v) A_5 cytoplasm derived from cultivated pigeonpea (*C. cajan*) (Mallikarjuna and Saxena 2005). Among these, A_4 cytoplasm was found to be the most efficient system of CMS for exploitation of commercially viable hybrid technology in pigeonpea (Saxena et al. 2009). In this present paper we report the development of an A_4 cytoplasm (*Cajanus cajanifolius)*-based CMS line and its heterotic restorer, resulting in a commercially viable hybrid (ICPH 2671) technology in pigeonpea (Saxena et al. 2013).

Materials and Methods

Development of CMS line

During 2003, a set of 43 crosses were made on the *Cajanuscajanifolius* (designated as A_4cytoplasm) based CMS line ICPA 2039 (Saxena et al. 2005) as female parent with 43 different accessions in glasshouse. The F_1s were then grown in field under net (mesh size 1 mm) to avoid insect-aided outcrossing in 4 row plots of 4 m row length during 2004. Standard agronomic practices (Saxena 2006) were followed to raise a healthy crop. Data were recorded on male sterility by examining five random, fully developed but unopened flowers of individual plants. Pollen load was checked by pollen smear test, and confirmed in the laboratory by squashing the anther in 2% acetocarmine. The fertile pollen absorbed stains and appeared round and dark purple under the microscope, while the sterile ones were shriveled and translucent. The number of fertile and sterile pollen was counted in three microscopic fields. Plants with <10% stained pollen were classified as male sterile. Sterile plants were covered with a muslin cloth bag (5 µm) to check for seed set (indicating male fertility). Single plant data were recorded on male sterility and other important morphological traits. Out of 43 crosses, the cross between ICPA 2039 and ICPL 20176 maintained male sterility and was designated as ICPA 2043 (Table 1). The recurrent parent (ICPL 20176) was designated ICPB 2043. The F_1 was backcrossed during the rainy season of 2003 to the male line ICPB 2043 on single plant basis under a net in the field. In the succeeding backcross generations (BC_2F_1- BC_6F_1) from 2004 to 2008 single plant selections for male sterility were done under a net in the field. ICPA 2043 in BC_6F_1 along with its maintainer

Table 1. Male sterility segregation of the CMS line ICPA 2043 developed through backcross breeding from the cross ICPA 2039 × ICPL 20176.

Generation	Year	Location	Number of plants		Male Sterility (%)
			Total	Male sterile	
F_0	2003	Glasshouse	-	-	-
F_1	2003	Field	18	18	100.00
BC_1F_1	2004	Field	71	67	94.37
BC_2F_1	2005	Field	99	92	92.93
BC_3F_1	2006	Field	648	634	97.84
BC_4F_1	2007	Field	711	707	99.44
BC_5F_1	2007	Field	145	145	100.00
BC_6F_1	2008	Field	197	197	100.00

line ICPB 2043 was grown in an open field during the 2008 rainy season to access phenotypic similarity of the ICPA 2043 and ICPB 2043 lines. From a total of 197 plants, 20 random competitive plants were selected for data recording of important qualitative and quantitative traits. Plant height (in cm) was recorded before harvesting, from base of plant to the highest point, and stem thickness (in mm) was recorded by a vernier caliper at 5 cm above ground. Data on seeds pod^{-1}, 100-seed weight, pods plant^{-1}, and seed yield were recorded at maturity. Student's t test was computed at 19 d.f. in 95% confidence interval limit, with two-tailed P values using the computer software program WINKS SDA 6.0.5 (TexaSoft 2007).

R-Line development

The restorer line was bred through a series of selections in the segregating generations of the cross C11× ICP 1-6-W3XWIX with pedigree ICPL 78143-WB-WB-WB-WB-W27-B. This line was screened for *Fusarium* wilt and sterility mosaic diseases from 2003 to 2008. Single plant selections were done for high fertility restoration. On the basis of performance of this line for 2 years (2004 and 2005) in terms of good standard heterosis, wilt and sterility mosaic resistance in a cross combination with ICPA 2043, the pollinator parent was designated as ICPR 2671.

Hybrid development and evaluation

The CMS line ICPA 2043 in BC_6F_1 generation was crossed with the restorer line ICPR 2671 to generate the F_1 hybrid (ICPH 2671) seed. The hybrid was evaluated for fertility restoration and other agronomic traits for 2 years (2004-2005) in station trials at ICRISAT, Patancheru (17°N). It was also tested in 36 environments (12 locations × 3 years) in the respective rainy seasons during 2006- 2008 in a two-replication complete randomized block design (RCBD) with four row plots of 4 m row length.

Results

Out of 43 crosses made in a glasshouse during 2003 with different accessions, all the 18 the F_1s of the cross ICPA 2039 × ICPL 20176 were found to be 100% male sterile (Table 1) during rainy season of 2004. ICPA 2043 and its maintainer ICPB 2043 were similar for most of the qualitative and quantitative traits (Table 2). ICPA 2043 has light yellow flowers (wing petal), green pods and stem, with lanceolate leaves, round brown seeds, and high wilt and sterility mosaic disease resistance. The mean plant height was 242.80 $\pm$4.87 cm with 8.60 $\pm$0.90 primary branches, 18.90 $\pm$1.79 secondary branches, 108.15 $\pm$0.22 days to 50% flower, 3.55 $\pm$0.06 seeds pod^{-1}, and stem thickness of 20.75 $\pm$0.68 mm. Paired t-test was performed to determine if there were differences between ICPA 2043 and ICPB 2043 lines for these traits. The mean differences between these lines were not significantly different from zero for all the traits at 95% confidence

Table 2. Comparison of important qualitative and quantitative traits of ICPA 2043 and its maintainer line ICPB 2043.

Character	ICPA 2043	ICPB 2043
Growth habit	Indeterminate	Indeterminate
Standard petal	Pale streak on dorsal side	Pale streak on dorsal side
Flower color	light yellow	light yellow
Pod color	Green	Green
Stem color	Green	Green
Seed coat color	Brown	Brown
Leaf shape	Lanceolate	Lanceolate
Wilt (%)	8.0	9.0
Sterility Mosaic (SM) (%)	0	0
Days to 50% flower	108.15 + 0.22	108.55 + 0.29NS
Days to maturity	169.05 + 0.23	159.10 + 0.24*
Plant height (cm)	242.8 + 4.87	248.05 + 2.74NS
No. of primary branches	8.6 + 0.90	8.95 + 0.94NS
No. of secondary branches	18.9 + 1.79	21.55 + 1.75NS
Stem thickness (mm)	20.75 + 0.68	22.35 + 0.47NS
Pod plant^{-1}	291.6 + 3.89	434.15 + 30.74*
Seeds pod^{-1}	3.55 + 0.06	3.67 + 0.07NS
100-seed weight (g)	11.50 + 0.03	9.35 + 0.06*
Pod weight (g)	166.17 + 14.35	227.18 + 14.62*
Seed yield plant^{-1} (g)	110.39 + 9.82	140.03 + 8.83*

*Significant difference in 95% confidence interval for Student's paired 't' test statistics.

interval with two-tailed *P* values 0.074≤*P*≤0.791, except for traits such as days to maturity (mean of 169.05±0.23 days), 100-seed weight (11.50±0.03 grams), pod weight (166.17 ±14.35 grams), seed yield plant^{-1} (110.39 ±9.82 grams), and pods plant^{-1}(291.6 ±3.89) for which ICPA 2043 was significantly different from that of ICPB 2043. Paired t-test results gave *P* values ranging from <0.001 to 0.030.

At ICRISAT Patancheru, a *Fusarium* wilt and sterility mosaic disease resistant restorer line (<10% wilt and sterility mosaic) designated as ICPR 2671 was found to restore male sterility perfectly (100%) for 2 years (2004-2005) in the station trials at ICRISAT, Patancheru (17°N). The mean fertility restoration across 36 environments for ICPH 2671 was 96.49%, with a range of 30-100% (Fig. 1). Except for Jalna (19.5°N), the mean fertility restoration for all the 11 locations (spanning 17-21°N latitude) was 97% or more.

Discussion

Development of CMS line

A stable A_4 (*C. cajanifolius*) cytoplasm based CMS line ICPA 2039 (Saxena et al. 2005) in BC_5F_1 was chosen as donor parent for CMS trait, rather than *C. cajanifolius* itself. This was done to avoid the undesirable linkage drag in terms of seed size, shape, color, seed coat thickness, and other unsuitable traits associated with wild species. The $F_{1s}$of the ICPA 2039 × ICPL 20176 cross were found to be 100% male sterile, and hence was chosen for introgression of A_4 CMS system in ICPL 20176, as this line behaved as a perfect maintainer. Presence of all male sterile plants in F_1 may be due to presence of recessive nuclear non-restorer gene (s) in ICPL 20176. This may also be due to small number of plants (18) tested. The male sterility percentage dropped to 94% in BC_1F_1, and gradually attained 100% male sterility again four generations later in BC_5F_1. The apparent drop in the maintenance percentage may be attributed to relatively larger population size (71, 99, and 648 in BC_2F_1, BC_3F_1, and BC_4F_1, respectively). It may also be due to presence of minor genes/ modifiers affecting male sterility. In each backcross generation, single plant selections were made for male sterility maintenance. This may have resulted in elimination of the modifiers responsible for male fertility, and the recessive nuclear gene(s) might have got fixed during the selection process. This is evident from the pollen shedder free segregation pattern in BC_5F_1 and BC_6F_1 generations in 2007 and 2008, respectively (Table 1). We therefore succeeded in introgressing *C. cajanifolius* orA_4 cytoplasm in ICPA 2043. ICPL 20176 is designated as its maintainer line ICPB 2043. Only wilt and sterility mosaic disease resistant progenies were selected for backcrossing with the recurrent parent. This resulted in high levels of wilt and sterility mosaic resistance in ICPA/B 2043.

Characterization of ICPA 2043 and ICPB 2043

Six generations of backcrossing of male sterile line, ICPA 2043 with the maintainer recurrent parent ICPB 2043 (ICPL 20176) resulted in more than 99% substitution of the nuclear genome of the CMS donor parent ICPA 2039, leading to development of ICPA 2043. While the A_4 CMS donor ICPA 2039 is an early-maturing determinate type line (Saxena et al. 2005), ICPA 2043 is a medium-maturating, non-determinate CMS line. While ICPA 2043 and its maintainer ICPB 2043 were similar for most of the qualitative and quantitative traits (Table 2), the differences for days to maturity, pod plant^{-1}, 100-seed weight, pod weight, and seed yield plant^{-1} may be attributed to the physiological deviations between ICPA 2043 and ICPB 2043 line. Being a male sterile line, pollination and pod setting in ICPA 2043 are dependent on

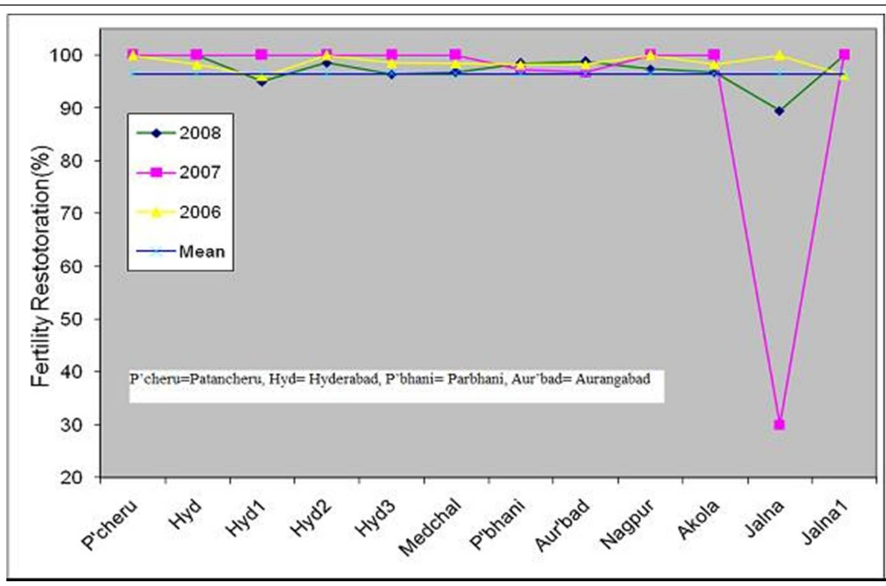

Fig. 1. Fertility restoration of ICPH 2671 at 12 different locations for three years.

insect-aided pollination, which essentially is a slow and continuous process. Pod setting, hence, becomes a function of time and frequency of insect visits, and stigma receptivity.

In pigeonpea fertilization must take place within 1-4 days after flower opening, since stigma receptivity remains high only till 20-120 hours (Luo et al. 2009; Prasad et al. 1977). Any late pollination results in lack of pod set. Hence it appears that delayed maturity (~10 days) of the ICPA 2043 line may be due to slow and (possibly) late pod setting. Similar results were noted by Saxena et al. (2005) in the CMS line ICPA/B 2039. This is in contrast to the maintainer line (ICPB 2043 line) where most of the pods are formed during the first flush, and therefore matures earlier. Lower pod (and seed) set may also be ascribed to the indeterminate nature of the ICPA 2043 line, in contrast to the determinate CMS line used in the study by Saxena et al. (2005) who reported a higher number of pods in the male sterile line. In non-determinate male sterile lines like ICPA 2043, it is difficult for the insect vector to pollinate all the flowers as these are spread along the length of the branches. Pollination (and seed set) is mostly limited to top and peripheral regions of the plant. In determinate lines, flowers are borne in clusters, and it becomes for the insects to gain access to all the flowers and transfer pollen from ICPB 2043 line, resulting in more pod and seed set. Higher 100-seed weight of ICPA 2043 may be linked to lesser pod load and gradual seed development process. In this case, the ICPA 2043 line is able to better cater to the needs of developing seeds with a lower number of pods/seeds, and the amount of maternal resources received per seed is higher (as the light harvesting area is comparable) than normal plants (ICPB 2043 line). Higher 100-seed weight may also be due to lower inter-seed competition within and between developing pods. This phenomenon is known to occur in plant kingdom where developing seed hinders development of other seeds within and between pods, especially if the developing sporophytes are half-sibs (Mohana et al. 2001). This is supported by the kin selection theory which postulates that closely related seeds (full-sibs) resulting from fertilization by a single paternal parent compete less with each other than seeds fertilized by different begetters (Kress, 1981; Mohana et al. 2001). However, a detailed physiological study needs to be done to fully understand this issue.

Presence of standard heterosis

Of all the 78 F_1s in cross combination made with ICPA 2043 during the rainy season 2003, the cross ICPA 2043 × ICPR 2671 was found to have the most promising standard heterosis. This cross was designated as ICPH 2671. In the multilocation yield trials conducted in South and Central India (4 years, 43 environments), ICPH 2671 reordered over 35% grain yield heterosis over popular cultivar Maruti (Saxena et al. 2009). This hybrid also recorded high levels of resistance to *Fusarium* wilt and sterility mosaic diseases, but was found to be susceptible to *Phytophthora* blight. In July 2008, ICPH 2671 was launched as 'Pushkal' by Pravardhan Seeds, a Hyderabad-based private seed company (Saxena 2009), and has been released in 2012 for the state of Madhya Pradesh, India. Some important characteristics of ICPH 2671 are presented in (Table 3).

Table 3. Important characters of ICPH 2671.

Characters	Mean value
Days to 50% flower	117 + 0.23
Days to maturity	172 + 0.69
Plant height (cm)	211 + 1.70
Pods plant^{-1}	298.85 + 19.55
Seeds pod^{-1}	3.57 + 0.04
100-seed weight (g)	11.64 + 0.03
Growth habit	Indeterminate
Branching pattern	Spreading
Stem color	Green
Flower color	Yellow with red streaks
Pod color	Purple
Seed color	Brownish purple
Leaf shape	Lanceolate

Fertility restoration of hybrid ICPH 2671

High degree of fertility restoration is a critical element in hybrid breeding for any crop species, since absence of out-crossing will not result in any seed set if fertility restoration is low. Hence, fertility restoration was tested for all the 78 F_1s in cross combination with ICPA 2043 during the rainy season 2003. Only such hybrids where fertility restoration exceeded 90% were selected for re-evaluation during 2004 rainy season. The unusually low fertility restoration at Jalna may be due to local microclimate with high relative humidity (particularly during the night), cloudy weather, and poor sunlight. These factors are known to adversely affect microsporogenesis, pollen production, and dehiscence (Duvick 1959). Overall, ICPH 2671 showed a high and stable fertility restoration over locations and years with no significant evidence for GxE interaction. Similar results were observed by Dalvi et al. (2008). High fertility restoration was as expected considering that the CMS cytoplasm is derived from the closest wild relative and the immediate progenitor, *C. cajanifolius* (De 1974), facilitating a favorable genome-plasmon interaction in which the gene product of the restorer gene compliments the defect present in the mitochondrial genome (Schnable and Wise 1998). It is known that generally restorer genes occur in those closely related gene pools where the male sterile genes are found. The ORFs/genes responsible for male sterility mostly reside in mitochondria (Liu et al. 2011; Sloan et al. 2012). High frequency and degree of fertility restoration may be attributed to presence of overriding genes/ORFs in the restorer lines (Schnable and Wise 1998), such as in ICPR 2671. Further studies are needed to dissect the molecular basis of fertility restoration in pigeonpea.

Conclusion

Through development of ICPH 2671, a proof-of-concept has been successfully demonstrated for exploitation of a commercially viable A_4-based hybrid technology with high fertility restoration and good standard heterosis. This most promising CMS system of pigeonpea can be further exploited by breeding seed parents with diverse nuclear backgrounds and their corresponding restorers with good fertility restoration and high specific combining ability for high and stable yields across various agroecologies of Asia and Africa. The A_4 *CMS*-based system can also help bring down cost of hybrid seed production by virtue of its stable cytoplasm over different photoperiod and temperature regimes, thereby making hybrid seed affordable to the poor and marginal farmers. This CMS system can be diversified to generate hybrids for all the broad maturity groups reported in pigeonpea, namely, super-early, extra-short, short, medium, and late durations.

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Conflict of Interests

The authors declare that they have no competing interests.

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