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Development of a new CMS system in pigeonpea utilizing crosses with *Cajanus lanceolatus* (WV Fitgz) van der Maesen

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Abstract Cytoplasmic male sterility is an important biological tool which is now available to pigeonpea breeders to exploit heterosis/hybrid vigor. A variety of CMS systems have been developed when wild relatives of pigeonpea from different gene pools were crossed as the female parent with cultivated types as the male parent. This paper reports a second source of CMS developed by using the cultivated pigeonpea as the female parent and one of its wild relative *Cajanus lanceolatus* (WV Fitgz) van der Maesen as the pollen donor, as such the A5 CMS system derived from *C. acutifolius.* All the F_1 hybrids were evaluated to confirm hybridity using 27 simple sequence repeat (SSR) markers. SSR marker analysis of parents provided 17 polymorphic markers from a total of 27 SSR markers used. Subsequently polymorphic SSRs were used to confirm the hybridity of the F_1 plants. F_1 hybrid plants were crossed with a range of pigeonpea cultivars to identify maintainers of male sterility. Morphology of the F_1 and backcross generations, cytology of the sterile as well as fertile floral buds derived from the crosses between sterile F_1 hybrids and unrelated pigeonpea cultivars were studied. An important observation made was that male sterility was a post meiotic process. Microsporogenesis was normal until the tetrad stage, but none of them formed pollen grains. Instead, they grouped together within the pollen mother cell wall and the tetrads did not separate into individual pollen grains.

Keywords CMS · *C. lanceolatus* · Cytology · Microtomy · Meiosis · Morphology · Pollen fertility · Sterility

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Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is an important food legume crop currently being grown on 5.2 million ha in the rain-fed areas of Asia, eastern and southern Africa, Latin American and Caribbean countries. Pigeonpea seeds are the primary source of protein for the vegetarian population (Saxena 2006). Pigeonpea is different from other legumes in being classified as an 'often cross-pollinated crop' with 25–70 % natural out-crossing reported from different locations (Saxena et al. 1990). Although, out crossing is an essential trait in hybrid breeding technology, it is not desirable in maintaining the genetic purity of cultivars in pigeonpea breeding as it creates variation, which may lead to reduced yields and elimination of

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disease resistance genes. To overcome this problem, cytoplasmic male sterility (CMS) system was developed (Saxena et al. 2010). Moreover, the CMS system is indispensable for pigeonpea, as its productivity was stagnant at 700 kg/ha for decades in spite of more than 50 varietal releases (Mallikarjuna et al. 2012). In India, the annual pigeonpea grain production of 2.74 million tones falls short of the domestic demand by about 0.5–0.6 million tonnes and the shortfall in production is imported from various countries across Asia and Africa (Mallikarjuna et al. 2012). In order to meet the ever growing demand for pigeonpea, its productivity must be increased by adopting new and appropriate technologies. So far, eight CMS systems have been reported in pigeonpea (Mallikarjuna et al. 2012; Saxena 2013). Of these, seven have been developed utilizing wild relatives from secondary gene pool (Saxena et al. 2010; Saxena 2013) and one from a tertiary gene pool of pigeonpea (Mallikarjuna et al. 2012). Amongst the eight CMS systems, only one system has cultivated pigeonpea cytoplasm (Mallikarjuna and Saxena 2005). In the present investigation, we report the development of a CMS system which has been derived from crosses involving pigeonpea cultivar as a female parent and a wild species, *Cajanus lanceolatus* (WV Fitgz) van der Maesen as the male parent. Confirmation of the production of F_1 hybrids was carried out using SSR marker analysis.

Materials and methods

Cajanus lanceolatus (ICP 15639) and *C. cajan* (ICPL 85010) plants were grown and crosses were made using *C. cajan* as the female parent and *C. lanceolatus* as the pollen donor. F_1 progenies were grown in a glasshouse in 25 × 25 cm plastic pots filled with sterilized Alfisol mixture (four parts Alfisols: two parts farm yard manure: one part sand). Alfisols is one of the major (native fertile) soil order in semi-arid tropics (Pathak et al. 2013). The individual plants were observed for male-sterility. Of these, pollen sterility was studied in five F_1 hybrids F_1 P-1, F_1 P-4, F_1 P-7, F_1 P-10 and F_1 P-12 using the standard acetocarmine test (Kaul and Singh 1969). The F_1 male-sterile plants were crossed to 11 unrelated pigeonpea cultivars namely MN1, MN5, MN8, ICPL 88039, ICPL 88034, ICPL 85030, ICPL 16198, ICPL 1447, ICP 14444, ICP 7035, ICP 92016 and a female parent cv. ICPL 85010, of which only seven cultivars (MN1, MN5, MN8, ICPL 88039, ICPL 85010, ICPL 88034 and ICPL 85030) were able to set the pods. Hand emasculation of sterile floral buds was followed by hand pollinations, using pollen grains of pigeonpea cultivars each day before 10 a.m. To retain the pollinated buds on the mother plant, gibberellic acid (50 mg/l GA3) was applied to the base of the pistil for three consecutive days after pollination.

Cytology

Flower buds were squashed in 4 % acetocarmine and well spread preparations were examined. To summarize different stages of meiosis namely metaphase, anaphase and tetrad, at least 20 pollen mother cells (PMCs) were examined. Pollen fertility analysis was carried out by staining mature pollen grains in 4 % acetocarmine. Well stained grains were counted as fertile grains and partial to unstained grains were counted as sterile. Alexander's stain (1969) was also used to count abortive and non-abortive pollen grains. Malachite green, a component of Alexander stain, stains the pollen walls green. Acid fuchsin, another component of Alexander's stain, stains the protoplasm red and hence it colors the non-aborted pollens red to deep red. As the aborted pollen grains are devoid of contents, they are stained green.

Microtomy

For microtomy, flower buds at the required stage were fixed in FAA (formalin:glacial acetic acid:absolute alcohol) solution and dehydrated through a series of tertiary butyl alcohol and xylol series and embedded in paraffin wax (Malikarjuna and Kalpana 2004). Preparations were stained in toluidine blue following the protocol of O'Brien et al. (1964).

SSR marker analysis

For molecular characterization of parents and hybrids, a total of 100 SSRs were selected from Bohra et al. (2011). Polymerase chain reactions (PCRs) for amplification of SSR loci were performed using thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) (Bohra et al. 2011) and the PCR products were checked for amplification on

1.2 % agarose gel. The amplification products, obtained using fluorescent dye-labeled primer pairs, together with Rox Gene Scan-500 labeled internal size standards, were analyzed on ABI 3730 Genetic Analyzer version 4.0 (Applied Biosystems, Foster City, CA, USA). Fragment analysis data were collected by the data collection software and pre-processed, converted to pseudogel images and further analyzed by the Gene Mapper software (Applied Biosystems, Foster City, California, USA).

Results

Crossability between sterile hybrids and pigeonpea cultivars

In comparison to the fertile plants, the anthers of male sterile plants were smaller in size and totally devoid of fertile pollen grains. On the contrary, the anthers from the fertile plants contained more than 50 % fertile pollen grains. Pod and seed set on the male-sterile plants after hand pollination indicated that the male-sterile plants had female fertility. Sterile F_1 hybrids were crossed with a range of pigeonpea cultivars. A maximum of 20.9 % pod set was observed in the cross F_1P-1 × ICPL 85010 and pods did not form when crossed with ICPL 88034 (Table 1). But when it crossed with ICPL 88039, ICPL 85010, MN1 and MN5 pod set ranged from 1.1 to 11.2 %. The trend of maximum pod formation when crossed with ICPL 85010 was also observed in crosses with three out of the other four male sterile plants (Table 1), the exception being the cross F_1P12 × MN8 where a maximum of 17 % pod set was observed.

Pollen fertility studies

Detailed pollen fertility studies were carried out using both acetocarmine and Alexander's staining techniques to further confirm sterility and fertility of the anthers (Fig. 4e, f, g). Anthers of partially fertile F_1 hybrids (P2, P3, P5, P6, P8, P9, P11, P13 and P14) exhibited (fertility 35–50 %) presence of both pink color stained fertile and green color stained sterile pollen gains as a result of staining them with Alexander's stain (Table 2). Whereas anthers from male sterile F_1 plants such as P_1, P_4, P_7, P_{10} and P_{12} showed all (100 %) the pollen grains stained green. Although the number of pollen sterile plants was lesser than the fertile plants, total sterility was observed in these plants. Anthers from such plants were small and shrunken. An important observation was that male sterility was post meiotic process.

In the BC_1F_1 plants obtained as a result of crossing between 100 % sterile F_1 hybrids and different pigeonpea cultivars, a range of pollen sterility (19–100 %) and partially fertile lines were obtained. BC_1F_1 hybrids from F_1P-4, obtained as a result of crossing cv. ICPL 85010 and MN1, resulted in sterile progeny lines along with partially fertile lines. However, crosses with other cultivars such as ICPL 88039, ICPL 88034, ICPL 92016 and MN5 produced partially to highly fertile progeny. When male sterile F_1P-12 was crossed with ICPL 88034, only fertile plants were obtained but with cultivars MN1, MN5 and MN8, ICPL 85010, ICPL 88039 both fertile and sterile progeny were obtained. Maximum number of sterile plants were seen in the cross with MN1 (Table 3).

Morphology of F_1 and BC_1 plants

F_1 hybrids were studied for morphological characters, meiotic behavior, pollen sterility and hybridity using SSR markers. Variation was observed with respect to flower morphology between the parents and the hybrids. The flower color was orange-yellow in the F_1 and BC_1 hybrids, whereas the cultivar and *C. lanceolatus* had yellow flowers. Hybrid flowers had dense streaks on their keel petal, the trait which was transferred from male parent (*C. lanceolatus*). In the female parent, flowers had no streaks on their keel petal. In comparison to the fertile plants, the anthers of male sterile F_1 plants were smaller in size and totally devoid of fertile pollen grains (Fig. 1a). Pods were flat in *C. lanceolatus* compared to *C. cajan* pods (Fig. 1b). The locules between the seeds were more prominent in F_1 and BC_1 hybrids (Fig. 1b) as in the male parent (*C. lanceolatus*) with clear cut demarcations between individual locules. Prominent differences in seed color were observed between the hybrids and the female parent. Seeds of *C. lanceolatus* were grayish black and cultivar seeds were beige-brown, while the BC_1F_1 seeds obtained through backcrossing the male sterile F_1s with cultivated pigeonpea were black in color (Fig. 1c) resembling the pollen parent. With respect to

Table 1 Percent pod set in sterile F_1 hybrids when crossed with different unrelated pigeonpea cultivars

Sterile hybrids	Pollinator	No. of pollinated buds	% of pod set
ICPL 85010 × ICP15639 F_1 P-1	ICPL 85010	320	20.9
	ICPL 88039	125	11.2
	ICPL 88034	78	0
	ICPL 85030	170	1.1
	MN 1	86	8.1
	MN 5	128	6.2
ICPL 85010 × ICP15639 F_1 P-4	ICPL 85010	239	30.9
	ICPL 88039	25	24
	ICPL 88034	25	16
	ICPL 92016	25	12
	MN 1	35	28.5
	MN 5	113	3.5
ICPL 85010 × ICP15639 F_1 P-7	ICPL 85010	343	22.7
	ICPL 88039	28	14.2
	ICPL 88034	30	13.3
	MN 1	40	22.5
	MN 5	68	1.4
ICPL 85010 × ICP15639 F_1 P-10	ICPL 85010	710	11.2
	ICPL 88039	136	2.2
	ICPL 88034	70	1.4
	ICPL 85030	145	2.7
	MN 1	159	3.7
	MN 5	160	5
	MN 8	65	1.5
ICPL 85010 × ICP15639 F_1 P-12	ICPL 85010	690	10.1
	ICPL 88039	235	1.7
	ICPL 88034	175	6.8
	ICPL 85030	162	0.6
	MN 1	200	6.5
	MN 5	150	9.3
	MN 8	80	17.5

plant height, all the F_1 hybrids resembled the male parent in being tall unlike the female parent (Fig. 2).

F_1 sterile hybrids (F_1P1, F_1P4, F_1P7, F_1P10 and F_1P12), when back-crossed to cultivated varieties (ICPL 85010, ICPL 88034, ICPL 88039, ICPL 85030, MN1, MN5, MN8 and ICPL 92010), produced 0–30 % of BC_1 pods (Table 2). A wide range of segregation was observed in BC_1 plants. All the BC_1 plants resembled the female parent i.e. cultivated pigeonpea being short statured than *C. lanceolatus.* There was segregation for flowering pattern in the BC_1F_1 population, showing 28 % determinate, 64 % to be semi determinate and 8 % of indeterminate (Fig. 3). More percentage (92 %) of BC_1F_1 population showed determinate and semi determinate flowering pattern as in the cultivated pigeonpea. Only 8 % of BC_1F_1 population resembled the male parent (*C. lanceolatus*) in the flowering pattern. Other traits such as size and shape of the leaf, streaks on standard petal, size and shape of seed, color of seed, hairiness of pods, streaks and color of pods were intermediate between two parents involved in the cross.

Cytology of sterile lines derived from the cross between sterile F_1 hybrid and pigeonpea cultivars

Meiotic analysis of PMCs of the F_1 hybrids formed 11 bivalents which were predominantly rings. Occassion-

Table 2 Percentage of Pollen fertility in F_1 hybrids derived from the cross *Cajanus cajan* (ICPL 85010) × *Cajanus lanceolatus* (ICP 15639)

S. no.	Plant number	% of pollen fertility
1	ICPL 85010 × ICPW15639 F_1P1	0 (STERILE)
2	ICPL 85010 × ICPW15639 F_1P2	37.8
3	ICPL 85010 × ICPW15639 F_1P3	35
4	ICPL 85010 × ICPW15639 F_1P4	0 (STERILE)
5	ICPL 85010 × ICPW15639 F_1P5	40.5
6	ICPL 85010 × ICPW15639 F_1P6	40.7
7	ICPL 85010 × ICPW15639 F_1P7	0 (STERILE)
8	ICPL 85010 × ICPW15639 F_1P8	56
9	ICPL 85010 × ICPW15639 F_1P9	36.06
10	ICPL 85010 × ICPW15639 F_1P10	0 (STERILE)
11	ICPL 85010 × ICPW15639 F_1P11	45
12	ICPL 85010 × ICPW15639 F_1P12	0 (STERILE)
13	ICPL 85010 × ICPW15639 F_1P13	50
14	ICPL 85010 × ICPW15639 F_1P14	48

ally, the number of bivalents was fewer. Univalents were also found and the average number of univalents per cell varied from 1 to 5. Meanwhile, multivalents (i.e. trivalents and tetravalents) appeared at a lower frequency, which ranged from 0 to 2. Normal bivalent formation in majority of the PMCs was an indication that there was good recombination between the parental genomes. Meiotic anaphase I showed 50–70 % of PMCs with normal disjunction and remaining 30–50 % with abnormal disjunction of chromosomes. At the tetrad stage, 100 % normal tetrads were observed in all hybrids except in P7 in which 6 % of tetrads contained micronuclei. Pollen fertility was found to vary between 35 and 50 % in fertile hybrids (P2, P3, P5, P6, P8, P9, P11, P13 and P14). In some F_1 hybrids (P1, P4, P7, P10 and P12), total male sterility was observed with all the anthers having 100 % empty pollen grains.

The cytological study was carried out to confirm the chromosome number and meiotic abnormalities (if any) in fertile and sterile BC_1F_1 hybrids derived from the crosses between sterile F_1 hybrid P12 and other cultivated pigeonpea. The results showed normal chromosome pairing in both the fertile as well as sterile hybrids with regular bivalent (n = 11) formation in metaphase I (Fig. 4a) and equal separation of chromosomes in anaphase I with no detectable chromosomal abnormalities.

At metaphase I, precocious separation of bivalents was observed in most cells followed by formation of laggards (Fig. 4b, c) in a small number of PMCs (pollen mother cells). Non-synchronization (Fig. 4b) of genomes of two parents (sterile F_1 hybrid and cultivated pigeonpea) at different meiotic stages was observed in 6.5 % of the PMC of sterile anthers. However remaining 94.5 % of the PMCs showed regular bivalent formation at metaphase I (Fig. 4a). At anaphase I, the separation was normal (11/11) in 90 % of the PMCs studied while 10 % showed unequal separation due to 1 or 2 laggards (Fig. 4c). Laggards seen at anaphase I may be the result of univalents from metaphase I, because their frequencies were comparable and there were 1–2 laggards in every observation. Regular 11–11 separation was seen in most of the cells at anaphase I. Normal tetrads were observed after telophase II (Fig. 4d). Unseparated tetrads were found in the post tetrad stages (Fig. 4e) in agreement with the mechanism of microsporogenesis in sterile F_1 hybrids. Degeneration of microspores after tetrad stage resulted in empty shrunken sacs inside anther lobe observed in sterile anthers (Fig. 4g). Microspores within anther lobes which had been stained with Alexander's stain absorbed only green color (Fig. 4f, h).

Anatomy of microsporogenesis in male sterile and fertile BC_1F_1 plants obtained from the cross F_1P12 × MN1

The study was undertaken to study the process of microsporogenesis in the male sterile and male fertile lines derived from (ICPL 85010 (*C. cajan)* × ICP 15639 (*C. lanceolatus*)) P12 × MN1 (*C. cajan*). For

Table 3 Segregation of male sterility in F_1 BC_1 plants derived from the crosses between three sterile F_1s and pigeonpea cultivars

Cross	Pollinator	% of pollen sterility in F_1 BC_1 plants											
		0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100	% of fertile plants	% of sterile plants
(ICPL 85010 × ICP15639) F_1 P-4	ICPL 85010	0	1	1	1	1	0	0	1	2	2	77	33
	ICPL 88039	0	1	1	0	1	1	1	2	2	1	90	10
	ICPL 88034	0	0	0	0	1	0	1	2	0	0	100	0
	ICPL 92016	0	0	0	0	0	0	0	1	1	0	100	0
	MN-1	0	0	0	1	3	2	0	0	0	3	77	33
	MN-5	0	0	0	0	0	0	0	1	0	1	50	50
(ICPL 85010 × ICP15639) F_1 P-7	ICPL 85010	0	0	2	0	2	1	2	0	0	0	80	20
	ICPL 88039	0	0	1	0	1	0	1	0	0	0	100	0
	ICPL 88034	0	0	0	0	1	2	1	0	0	0	100	0
	MN-1	0	0	0	1	0	0	3	1	0	3	62.5	37.5
	MN-5	0	0	0	2	1	0	0	0	0	0	100	0
(ICPL 85010 × ICP15639) F_1 P-12	ICPL 85010	0	0	0	2	5	5	3	2	2	1	95	5
	ICPL 88039	0	0	0	0	0	0	2	2	0	1	80	20
	ICPL 88034	0	0	0	0	3	1	2	2	0	0	100	0
	ICPL 85030	0	0	0	0	0	0	0	0	0	1	0	100
	MN-1	0	0	0	1	3	2	2	2	1	7	61.1	38.8
	MN-5	0	0	0	0	3	0	1	4	2	1	90	10
	MN-8	0	0	0	0	1	2	2	0	0	1	83	17

Fig. 1 Morphology of diadelphous anthers pods and seeds. **a** *Yellow* fertile anthers of *Cajanus cajan* (L.) Millsp (cv. ICPL 85010—*left*), *white* sterile anthers of completely male sterile F_1 hybrid plant (*middle*) and *yellow* anthers of partially fertile F_1 hybrid (*right*). **b** Morphology of pods in parents and BC_1F_1 hybrid (*Cajanus cajan* (cv. ICPL 85010) on the *left*, *C. lanceolatus* (ICP15639) on the *right* and in the center are the BC_1 pods produced on male sterile F_1 hybrid. **c** Seed color in parents and BC_1F_1 hybrid (*Cajanus cajan* (cv. ICPL 85010) on the *left* and *C. lanceolatus* (ICP15639) on the *right* and in the center are the BC_1 seeds produced on male sterile F_1 hybrid. (Color figure onlie)

Fig. 2 Comparison of F_1 hybrids (*B*, *C*, *D*, *E* and *F* plants) with the female parent *Cajanus cajan* (*A*) and male parent *Cajanus lanceolatus* (*G*)

this study, one fertile and one sterile individual of F_1BC_1 generation was selected. These individuals were generated by crossing the sterile F_1P12 (female parent) with cultivated pigeonpea MN1 (male parent). The progeny resulted from this cross (P12 × MN 1) included both male sterile as well as male fertile plants. To confirm the sterility of the plant, anthers of sterile individuals were stained with Alexander's stain which showed sterile pollen stained completely green after 48 h of incubation. These flower buds of sterile individual plants were fixed for microtome sectioning to study the anatomy of microsporogenesis of male sterile plants.

Longitudinal section (L.S) and transverse section (T.S) of anthers of similar sizes from both male-fertile (Fig. 5a, b) and sterile (Fig. 5j, k) plants showed no

Fig. 3 Raceme morphology observed in the BC_1F_1 population derived from the crosses between Male sterile F_1 hybrids and cultivated pigeonpea. **a** Determinate growth habit. **b** Semi-determinate growth habit. **c** Indeterminate growth habit

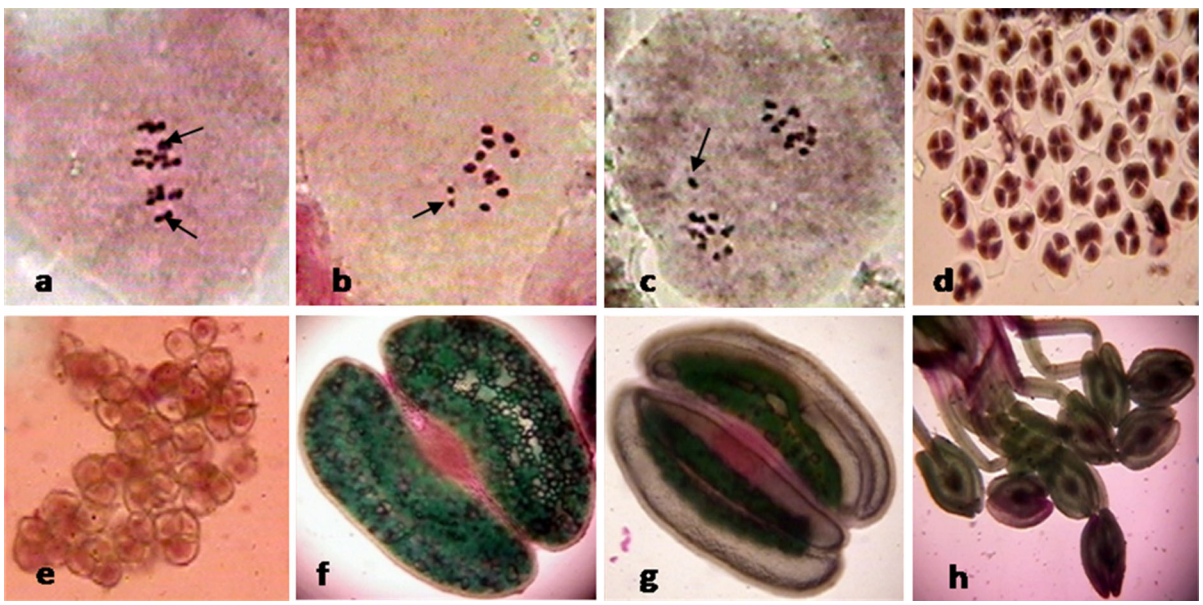


Fig. 4 Male meiosis in male-sterile BC_1F_1 hybrid derived from the cross between sterile F_1 hybrid and pigeonpea cultivar [ICPL 85010 (*Cajanus cajan*) × ICP15639 (*Cajanus lanceolatus*)] F_1 × MN1 (*Cajanus cajan*). **a** Normal metaphase with 11 bivalents (The *top arrow* points at the ring bivalent and the *bottom arrow* points at the rod bivalent). **b** Metaphase with 2 univalents (*arrow*). **c** Anaphase I with one laggard (*arrow*). **d** Normal tetrads. **e** Tetrads with persistent tetrad wall. **f** Anther stained with Alexander's stain showing sterile *green* contents. **g** Degenerated microspores inside the anther at anthesis. **h** Sterile anthers devoid of pollen. (Color figure online)

differences in the development of sporogenous tissue. There were no differences between male fertile (Fig. 5c–g) and male-sterile (Fig. 5n–p) anthers for the number of PMCs. The anther walls of both fertile and sterile types consisted of four distinct layers; an epidermis (E), an endothecial layer (En), a middle layer and a tapetal layer (T) (Fig. 5c–l). First nuclear divisions of tapetal cells occured before meiosis.

During the meiosis I, there was no change in the epidermis and endothecium. Even at the tetrad stage, T.S. of anther lobes showed normal tetrads in both male-fertile (Fig. 5c) and sterile (Fig. 5l) hybrids. In the case of fertile anthers, the process of microsporogenesis proceeded normally with the development of pollen grains (Fig. 5c–f). In fertile hybrids, the tapetal layer degenerated, thus nourishing the microspores until the formation of mature pollen grains (Fig. 5e–i). But in the subsequent stage, pollen grains were normal in male fertile (Fig. 5h, l) plant whereas shrunken in male sterile hybrid (Fig. 5m–r).

Both male-fertile and male-sterile plants proceeded normally up to the tetrad stage, and during this period, the tapetum in the male-sterile plants remained intact. 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. At the end of microsporogenesis in the sterile plants, only empty anther lobes were present enclosing the remnants of degenerated pollen (Fig. 5q, r).

Hybridity testing using SSR markers

In order to assess the hybridity of the eight F_1 hybrids (P1, P2, P4, P7, P8, P9, P10 and P11), developed by crossing *C. cajan* (ICPL 85010) × *C. lanceolatus* (ICP 15639), a total of 100 SSR markers were used. Out of these 100 SSRs, 27 SSRs could be amplified within the expected range of sizes (Table 4). Among these amplified primers, a total of 17 were found to be polymorphic between the parental genotypes and produced clear, scorable and unambiguous polymorphic bands. These 17 polymorphic SSR markers (CcM0008, CcM0035, CcM0047, CcM0057, CcM0710, CcM0974, CcM1991, CcM1459, CcM2012, CcM2071, CcM2176, CcM2228, CcM2505, CcM2639, CcM2672, CcM2707 and CcM2855) were used for the identification of the true hybrids. Out of 17 markers, 9 markers (CcM0047, CcM0057, CcM0710, CcM0974, CcM1991, CcM2012, CcM2228, CcM2505 and CcM2639; bold in Table 4) were found able to detect F_1 hybrids showing the heterozygotic condition of the hybrid/s (Fig. 6), whereas remaining seven markers (CcM0008, CcM0035, CcM1459, CcM2176, CcM2672, CcM2707 and CcM2855) produced either female or male specific alleles in F_1s. As a result all 8 F_1s were identified as true hybrids.

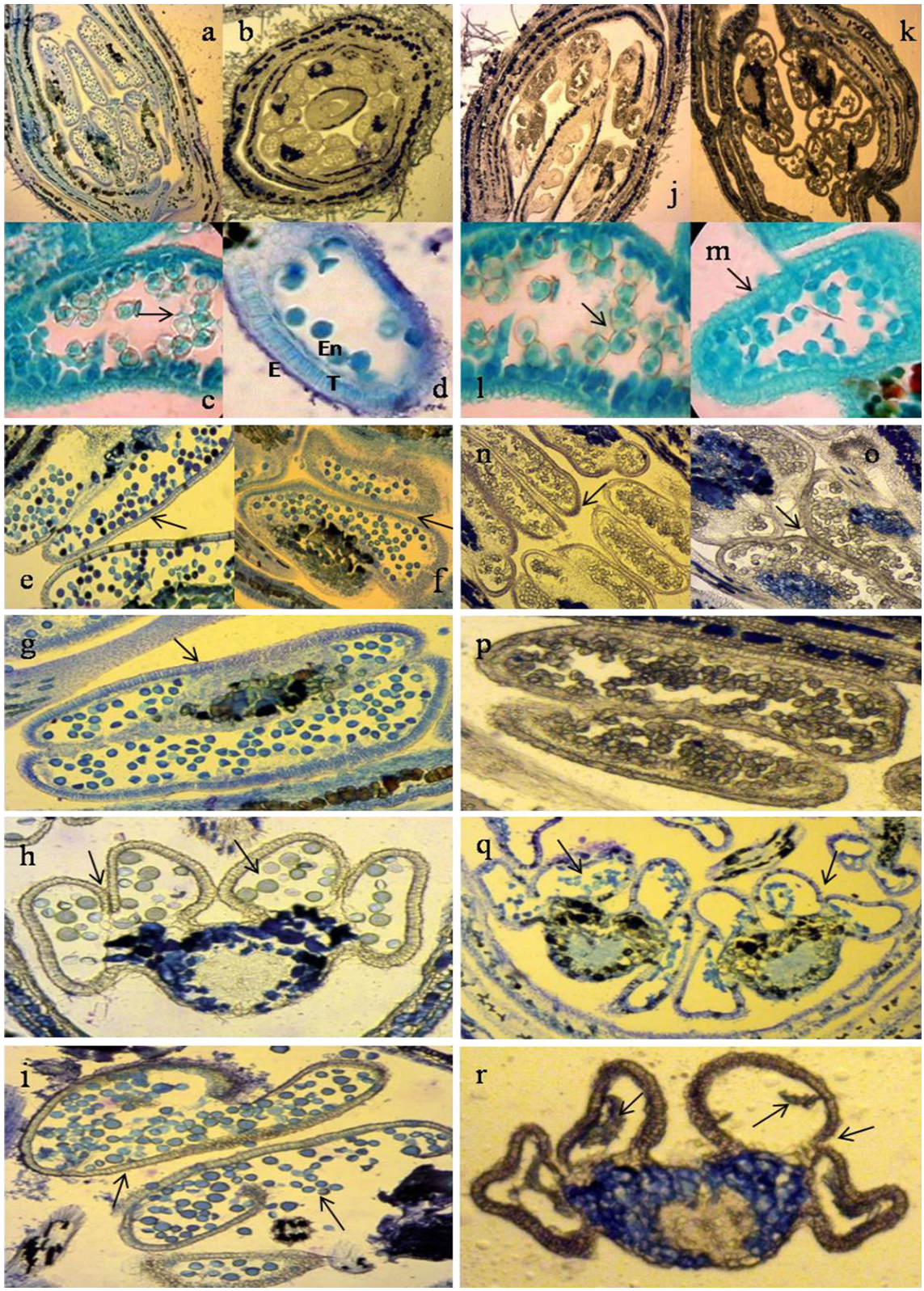
Discussion

With stagnancy in pigeonpea yield, it has become necessary to look for avenues to increase yield by the utilization of CMS system. Legumes in general were not good candidates until now for the utilization of CMS systems due to cleistogamous nature of flowers that does not permit economical mass pollen transfer. However pigeonpea is an exception with natural outcrossing up to 70 % (Saxena et al. 1990). In one of the CMS system (A7; Mallikarjuna et al. 2011), chasmogamous flowers were observed which is ideal for cross pollination. There are various CMS systems now available for pigeonpea (Saxena et al. 2010; Mallikarjuna et al. 2012) and some of the systems are being actively utilized to increase pigeonpea yield. For long term viability of a hybrid breeding system, diversification of both genetic as well as cytoplasmic system is essential. The present source of CMS being reported, which has to be developed further, offers good opportunities in this direction. The fertile plants obtained, are being advanced further to study alien introgression and presence of useful traits (Srikanth et al. 2013).

There are already 8 CMS sources reported (Saxena et al. 2010; Kumar and Saxena 2013) and amongst which, one has been reported on cultivated pigeonpea cytoplasm with the nuclear genome of *C. acutifolius* (A_5 CMS; Mallikarjuna and Saxena 2005). The present system will be named the A_9 CMS system. This system is the second CMS system being reported on cultivated pigeonpea cytoplasm, with nuclear genome of *C. lanceolatus*.

The results have shown that one of the male sterile lines F_1P12 produced only male sterile plants when crossed with cultivar ICPL 85030 and this cultivar can be classified as a good maintainer. The rest of the cultivars had different levels of fertility restoring abilities, especially ICPL 88034 produced only fertile plants and this variety can be classified as a good restorer. Utilization of many more and diverse pigeonpea cultivars in the crossing program will give rise to more number of restorers and maintainers. It is important to have more and diverse number of restorers to effectively exploit heterosis in pigeonpea.

Pollen fertility of the CMS lines showed that meiosis proceeded normally till the end of the divisional stages and during the formation of tetrads. Sterility mechanisms observed were due to interaction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◀**Fig. 5** Microtomy sections of flower buds from male-fertile and sterile BC_1F_1 hybrids derived from the cross [(ICPL 85010 (*Cajanus cajan*) × ICP15639 (*Cajanus lanceolatus*)] F_1 P12 × MN1 (*Cajanus cajan*). **a–i** Fertile anthers sections. **a** Longitudinal section (L.S) of fertile bud at pre-anthesis stage. **b** Transverse section (T.S) of fertile anther. **c** Sporangium with tetrads. **d** Anther wall layers showing epidermis, tapetum and inner wall layer. **e** Epidermis (E), tapetum (T) and endothecium (En) around PMCs. **f** Disintegrating tapetal layer at anthesis. **g** L.S of anther showing mature pollen. **h** T.S of anther showing fertile pollen. **i** Pollen grains inside the anther at anthesis. **j–r** Sterile anther sections. **j** L.S of sterile bud at pre-anthesis stage. **k** T.S of sterile anther at pre-anthesis stage. **l** Sterile anther lobe showing normal tetrads. **m** Sterile anther lobe showing tapetal layer. **n** and **o** Showing thick prominent tapetum at late tetrad stage. **p** L.S of sterile anther at staminate stage showing intact tetrads. **q** T.S of sterile anther with degenerating tetrads. **r** Sterile anther shriveled and indehiscent at anthesis

between the nuclear and cytoplasmic genomes of the parents involved in the cross and it was a post meiotic process and not due to parental genome incompatibility. In interspecific crosses involving distant genomes, abnormalities (univalents and multivalents) observed in metaphase and anaphase divisional stages are due to incompatibility between the nuclear genomes of the parents involved in the cross leading to pollen sterility. Such pollen sterility observations were made in the cross involving *C. platycarpus*, an incompatible wild relative of pigeonpea, placed in its tertiary gene pool (Mallikarjuna et al. 2011). Similarly, presence of univalents (1–5) and multivalents (0–2) resulted in partial sterility in fertile F_1 hybrids (P2, P3, P5, P6, P8, P9, P11, P13 and P14). Morphologically, F_1 hybrids showed the traits of both the parents in having tall nature of the male parent and with black seed coat color. With respect to flower color and size, it resembled the female parent. Hence, it was possible to identify the hybrids morphologically from the female parent.

In the CMS lines, it was observed that there was breakdown of the microsporogenesis associated with persistent tapetum observed in genetic male sterility reported by Reddy et al. (1978) and the non-dissolution of the tetrad wall as observed by Mallikarjuna and Saxena (2005) in the A_5 CMS system of pigeonpea. Mallikarjuna and Kalpana (2004) reported two types of microsporogenesis mechanisms in CMS lines. Type I CMS had partially or totally brown and shriveled anthers and the process of microsporogenesis was inhibited at the pre-meiotic stages. Type II CMS had pale, white, shriveled anthers and the break down in microsporogenesis was at the post meiotic stage after the formation of tetrads caused sterility of plants. The second one appeared to be the case with CMS in the present study, implying the interaction of the cytoplasmic genome of the female parent with the nuclear genome of the male parent to be responsible for pollen sterility. The post meiotic breakdown of microsporogenesis was also reported in *Allium cepa* (Virnich 1967).

Further evidence for post-meiotic breakdown of products of meiosis, was observed in anatomical studies of microsporogenesis. Tapetal layer provides nutrition to the developing microspores and degenerates as the PMCs mature into pollen grains. Microtomy of the developing anthers of sterile anthers showed a persistent tapetal layer when the PMCs formed tetrads. The tetrads continued to hold together in the tetrad wall without separating into individual pollen grains. This showed that the tapetum did not play any role in the development of the pollen grains whereas in the anthers from the cultigen, there was dissolution of the tapetal layer and development of individual pollen grains. Nine SSR markers were able to clearly detect hybrids, although there were other SSR markers which showed monomorphic bands. This may be due to the similarity of parents in those regions of the SSR markers. Bhora et al. (2011) have proved SSR markers developed for hybrid purity assessment in pigeonpea can be a powerful tool to efficiently detect true hybrids. In the present investigation too, SSR were a handy tool to detect hybrids in the early stage of development.

Utilizing other CMS systems which were developed on wild species cytoplasm takes longer to stabilize the CMS system. Hence the A_9 CMS system may have an added advantage in having the cultivated cytoplasm similar to the cytoplasmic genome of cultivated pigeonpea.

Synthesis of CMS systems by Saxena et al. (2010, 2013) and Mallikarjuna et al. (2012) have shown that wild relatives of pigeonpea, from both secondary and tertiary gene pool are good sources to develop CMS systems in pigeonpea, if concerted efforts are directed to develop CMS systems.

Table 4 Polymorphism status of SSR markers tested on eight F_1 hybrids derived from the cross *C. cajan* (ICPL85010) × *C. lanceolatus* (ICP15639) and two parental genotypes

S. No	SSR markers amplified	PARENT1 ICPL85010	PARENT2 ICP15639	Allele size (bp) in F_1 hybrids															
				P-1		P-2		P-4		P-7		P-8		P-9		P-10		P-11	
1	CcM0008	195.93	202.46	202.28	NA	194.33	203.19	202.02	NA	202.12	NA	194.64	203.41	194.71	NA	202.06	NA	194.57	203.27
2	CcM0035	275.95	275.07	275.25		275.91		275.07		275.66		275.07		275.12		275.07		275.17	
3	**CcM0047**	**174.91**	**185.36**	**174.92**	**185.45**	**174.84**	**185.46**	**174.94**	**185.55**	**175.06**	**185.53**	**175.09**	**185.60**	**175.06**	**185.51**	**175.02**	**185.65**	**175.00**	**185.61**
4	**CcM0057**	**290.81**	**283.78**	**284.01**	**290.9**	**283.49**	**290.63**	**283.8**	**290.79**	**283.69**	**290.75**	**284.08**	**291.19**	**284.04**	**290.95**	**284.32**	**291.52**	**284.1**	**291.51**
5	CcM0252	246.55	NA	246.94	255.37	242.70	253.28	246.84	257.07	246.74	257.17	242.45	253.04	241.98	253.44	246.77	257.77	243.35	253.53
6	CcM0416	147.33	NA	147.15	154.62	147.34	155.61	147.64	154.66	147.37	154.32	148.96	154.54	147.16	154.87	147.12	154.75	147.37	154.74
7	**CcM0710**	**297.06**	**286.99**	**287.09**	**297.21**	**287.16**	**297.18**	**287.05**	**297.05**	**287.09**	**297.21**	**287.12**	**297.06**	**287.08**	**297.14**	**286.80**	**297.01**	**286.73**	**297.09**
8	**CcM0974**	**175.31**	**169.23**	**179.20**	**171.90**	**175.19**	**172.08**	**179.32**	**171.85**	**179.22**	**171.47**	**175.34**	**167.79**	**175.44**	**168.19**	**179.69**	**168.23**	**175.58**	**167.89**
9	CcM0988	247.70	NA	245.78	252.04	240.94	248.03	245.14	252.14	244.93	253.87	240.43	246.82	240.56	250.18	244.71	254.01	240.94	250.27
10	CcM1385	272.26	NA	272.05	NA	271.99	NA	271.88	NA	272.06	NA	271.98	NA	271.96	NA	272.15	NA	272.17	NA
11	CcM1447	281.89	NA	281.66	NA	281.65	NA	281.71	NA	284.67	NA	281.76	NA	281.94	NA	281.58	NA	281.71	NA
12	CcM1459	183.52	183.40	183.14		183.10		183.40		183.46		183.46		183.19		183.83		183.76	
13	**CcM1991**	**208.52**	**194.55**	**194.34**	**206.3**	**194.47**	**206.51**	**194.98**	**205.92**	**194.45**	**206.18**	**194.14**	**208.02**	**194.34**	**208.29**	**194.51**	**208.53**	**194.38**	**208.41**
14	CcM1999	173.53	NA	173.24	NA	173.44	NA	173.22	NA	173.52	NA	173.47	NA	173.44	NA	173.41	NA	173.17	NA
15	**CcM2012**	**240.26**	**236.40**	**236.31**	**240.02**	**236.29**	**239.85**	**236.24**	**239.95**	**236.21**	**239.98**	**236.27**	**239.81**	**236.34**	**240.02**	**237.33**	**240.92**	**237.09**	**240.80**
16	CcM2066	119.23	NA	121.01	NA	119.14	NA	121.24	NA	121.16	NA	119.42	NA	119.50	NA	120.85	NA	118.97	NA
17	CcM2071	199.32	215.98	215.87	NA	217.93	NA	215.99	NA	215.91	NA	217.87	NA	217.93	NA	215.77	NA	217.78	NA
18	CcM2095	246.17	NA	242.22	NA	242.24	NA	242.07	NA	242.09	NA	242.30	NA	242.39	NA	242.07	NA	242.16	NA
19	CcM2176	273.09	271.80	271.93	NA	271.89	NA	272.05	NA	271.64	NA	272.29	NA	272.76	NA	272.03	NA	272.88	NA
20	**CcM2228**	**289.07**	**311.98**	**289.03**	**311.19**	**288.82**	**311.21**	**289.22**	**311.75**	**288.99**	**311.89**	**289.15**	**311.89**	**288.90**	**312.08**	**289.14**	**312.31**	**288.88**	**311.91**
21	CcM2379	165.11	NA	165.45	NA	168.20	NA	168.12	NA	167.51	NA	165.47	NA	165.45	NA	165.80	NA	167.86	NA
22	CcM2451	183.37	NA	183.44	NA	183.30	NA	183.30	NA	183.30	NA	183.53	NA	183.73	NA	183.50	NA	183.60	NA
23	**CcM2505**	**217.00**	**228.60**	**217.16**	**228.70**	**216.99**	**228.76**	**217.06**	**228.60**	**217.23**	**228.69**	**217.36**	**228.75**	**217.14**	**228.68**	**217.16**	**228.76**	**217.10**	**228.60**
24	**CcM2639**	**145.78**	**165.98**	**145.44**	**166.33**	**145.65**	**166.37**	**145.67**	**166.21**	**145.66**	**166.48**	**145.74**	**166.19**	**145.82**	**166.43**	**145.40**	**166.06**	**145.49**	**165.97**
25	CcM2672	243.72	243.98	243.66		243.66		243.55		243.83		243.69		243.83		243.80		244.01	
26	CcM2707	239.49	242.73	238.24	NA	242.70	NA	238.20	NA	238.24	NA	242.67	NA	242.76	NA	237.63	NA	241.44	NA
27	CcM2855	282.46	270.56	282.46	NA	270.03	NA	270.03	NA	270.07	NA	270.15	NA	270.03	NA	270.21	NA	270.22	NA

Primers and peak values in bold font representing the polymorphism in parents and hybrids

NA not amplified

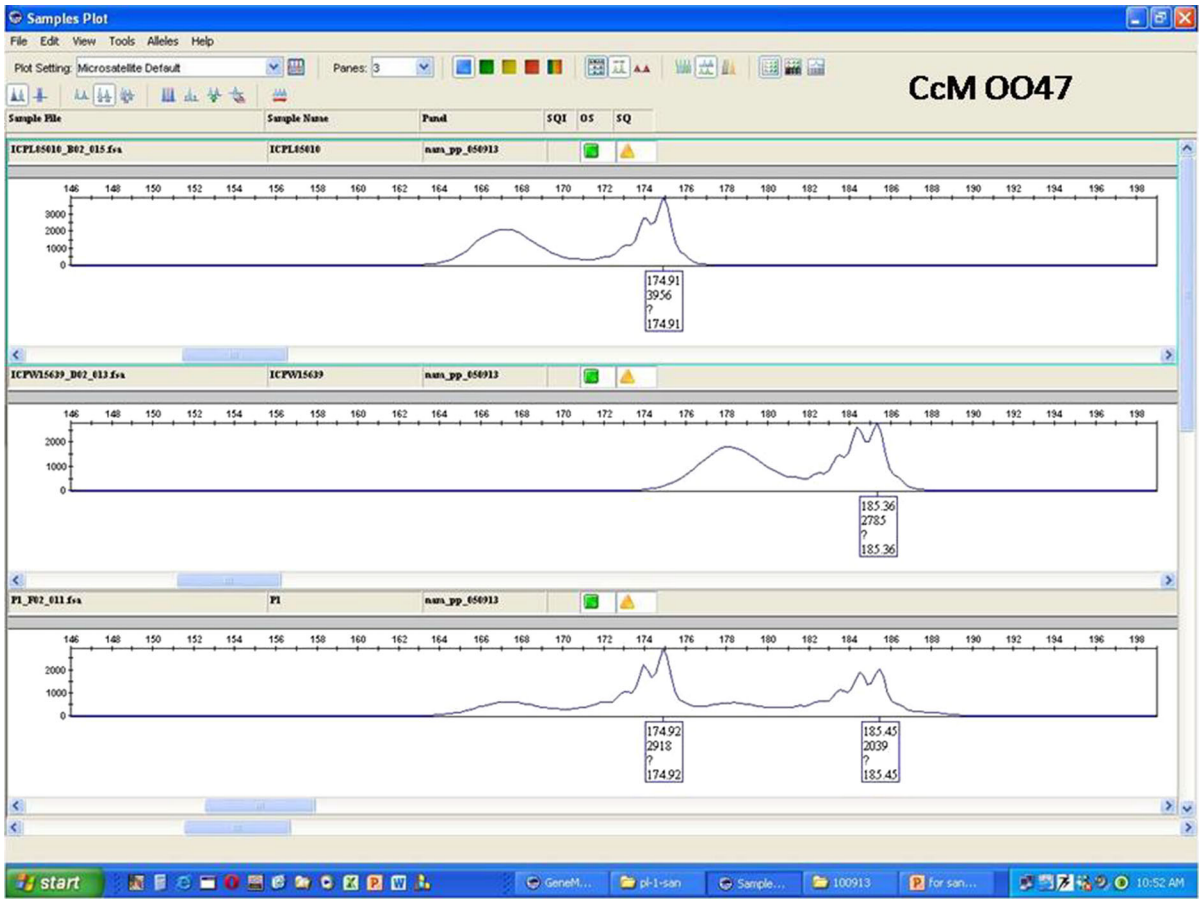

Fig. 6 Electropherogram of SSRs obtained with software Genemapper using the SSR marker Ccm0047. The *top line* is ICPL 85010 (*Cajanus cajan* (L.) Millsp, *second line* is ICP15639 (*Cajanus lanceolatus* (WV Fitgz) van der Maesen) and *third line* is F_1 hybrid

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