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Cajanus platycarpus (Benth.) Maesen as the donor of new pigeonpea cytoplasmic male sterile (CMS) system

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Abstract *Cajanus platycarpus*, a distantly related wild species, was successfully crossed with cultivated pigeonpea using embryo rescue and tissue culture techniques. Advance generation lines showed a range of desirable characters including cytoplasmic male sterility. A range of pigeonpea cultivars restored fertility and was maintained by a few lines including cultivar ICPL 85010. Clasmogamous flowers were observed in the male sterile lines. In such flowers anthers did not form di-adlephous bundle. Cytological analysis revealed that meiosis proceeded normally till the tetrad stage in those anthers with pollen grains. After which many of the pollen grains turned sterile. In the anthers with pollen grains, dehiscence was not observed, thus creating functional sterility. In many other anthers, pollen mother cells (PMCs) were not formed at all, giving rise to sepalous anthers. In conclusion two mechanisms of male sterility existed, one was premeiotic, where PMCs did not form and in the second, although PMCs gave rise to pollen grains, they were either partially or totally sterile accompanied by non-dehiscence of anther wall.

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Introduction

Pigeonpea (*Cajanus cajan* (L.) Millspaugh) is an important grain legume of Asia and Africa. In both the continents yield is an important trait to meet the protein needs in the diet of the people. It is often said that pigeonpea has reached its performance plateau (Saxena 2008), with a huge gap between the potential and actual yield. Since the land under cultivation is limited, yield of the crop is crucial. Release of more than 100 pure lines has not increased the productivity substantially (Singh et al. 2005), hence there is a need to look for other avenues to increase productivity. Unlike many other grain legume crops, pigeonpea is unique with the availability of cross pollination mechanism and its utilization to develop cytoplasmic male sterile (CMS) systems.

Research on the development of CMS system is less than two decades old, and a CMS system to exploit heterosis is already available (Saxena et al. 2005). The A_1 cytoplasm was developed with *C. sericeus* cytoplasm (Ariyanayagam et al. 1993, 1995). The system could not consistently maintain male sterile plants due to the presence of pollen shedders in the female parent and reversal of male sterile to fertile plants under low temperatures. Later Saxena (2005) carried out additional hybridization

and selection of male sterile plants. These male sterile lines reverted back to full fertility under low temperatures and shorter days and expressed complete male sterility under high temperature and longer days. Due to these drawbacks, the system is not in use. The A_2 cytoplasm derived from *C. scarabaeoides* (Tikka et al. 1997) is stable but fertility restoration is not consistent. Hence has not been developed further. The A_3 cytoplasm derived from *C. volubilis* (Wanjari et al. 2001) does not have any fertility restoration systems. Hence much work needs to be done before this system can be used. The currently in use A_4 cytoplasm derived from *C. cajanifolius* (Saxena et al. 2005) is the best available source due to its stability across environments and years. It is not desirable to depend on single source of CMS and diversification of CMS system is advisable. Research efforts led to the identification of A_5 cytoplasm with *C. acutifolius* as the pollen donor (Mallikarjuna and Saxena 2005). In this system cultivated pigeonpea was used as the female parent and crossed with wild species *C. acutifolius* and 100% male sterile lines were selected from this cross. In all the previously mentioned CMS systems, wild species were used as the female parent (*A. sericeus* in A_1 system; *C. scarabaeoides* in A_2 system; *C. volubilis* in A_3 system and *C. cajanifolius* in A_4 system). Research efforts to identify multiple maintainers and restorers of A_5 system is in progress. A_6 system with *C. lineatus* cytoplasm is under initial stages of development (Saxena et al. 2010).

In this report the A_7 CMS derived from *Cajanus platycarpus*, a wild species in the tertiary gene pool of pigeonpea (Mallikarjuna et al. 2010), is being reported. It is cross incompatible with the cultigen, which means F_1 hybrids add BC_1 progeny and cannot be produced by normally applicable hybridization techniques. Hormone aided pollinations coupled with embryo rescue techniques were utilized to obtain F_1 and BC_1 progeny. BC_1 plants set mature seeds (Mallikarjuna 2007). A selection was made in BC_2 progeny and called it BC_2-E line (Mallikarjuna et al. 2010). Majority of the plants in this line showed low pollen fertility. BC_2-E line was crossed with cv. ICPL 85010 and progenies with different levels of pollen fertility were obtained. In two lines viz., E4 and E15, all the pollen grains were sterile. Crosses were carried out between male sterile E15 with different pigeonpea cultivars/genotypes and the results showed that many of them were restorers of fertility but cv. ICPL 85010 maintained male sterility. Thus a new CMS system is being reported in this paper.

Materials and methods

BC_3 progenies between *C. platycarpus* and *C. cajan* cv ICPL 85010 called BC_3-E15 and BC_3-E4 and pigeonpea cultivars ICPL 85010, ICPL 1444, ICPL 87119 and ICPL 88014 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). Hand emasculation of BC_3-E15 and BC_3-E4 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 GA_3) was applied to the base of the pistil for three consecutive days after pollination.

Flower buds were squashed in 2% aceto carmine 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 2% aceto carmine. 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 stain, stains the protoplasm red and hence it colors the non-aborted pollens from red to deep red. As the aborted pollen grains were devoid of contents, they were stained green.

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. Preparations were stained in toluidine blue following the protocol of O'Brien et al. (1964).

Results

Hormone aided pollinations coupled with embryo rescue techniques gave rise to F_1 and BC_1 progeny

which are already reported in different publications (Mallikarjuna 2003, 2007). BC_1 plants produced mature seeds. A selection was made in BC_2 population for male sterility (Fig. 1). All the plants in this line called BC_2F_1-E had low pollen fertility. Pollen fertility in the lines ranged from 5 to 48%. Crosses between BC_2F_1-E and ICPL 85010 gave rise to two plants with total pollen sterility namely BC_3-E15 and BC_3-E4. These two plants were crossed with cultivars Asha, ICPL 85010, ICP 1444, UPAS 120 and *C. platycarpus* accession ICPW 68. The results of the crossing program are presented in Table 1.

Clasmogamous flowers (Lord 1981) were observed for the first time in BC_2-E plants and the trait persisted in BC_3 plants (Fig. 2) and also when these plants were crossed with other pigeonpea cultivars. Unlike in cultivated pigeonpea (Fig. 2a), the petals do not conform to a legumenaceous flower structure. The petals were separated and gave the appearance of a rubiacious flower (Fig. 2b). Anthers did not form a di-adlephous bundle (Fig. 2c, e) as seen in cultivated pigeonpea (Fig. 2d), with the separation of individual filaments.

Crosses between BC_3-E15 and cultivar ICPL 85010 gave rise to 7% pod set. In the first set, 18 plants were grown, their anther morphology studied and pollen fertility checked. Pollen fertility in the plants ranged from 0 to 3%. In the second set a few more plants were obtained and pollen fertility in them did not exceed 20% (Table 1). Most of the anthers were sepalous (Fig. 3d), transparent and devoid of a regular anther cavity and pollen grains. Some of the anthers had a miniature anther cavity with a few pollen grains (Fig. 3b, c). In some of the anthers, a few pollen grains appeared to be fertile, staining pink in acetocarmine. Staining them with Alexander's stain showed them to be stained green, reflecting abortiveness of the grains (Fig. 3e, f). Dehiscence of the anthers did not take place in any of the anthers to release the pollen grains. Upon closer study it was

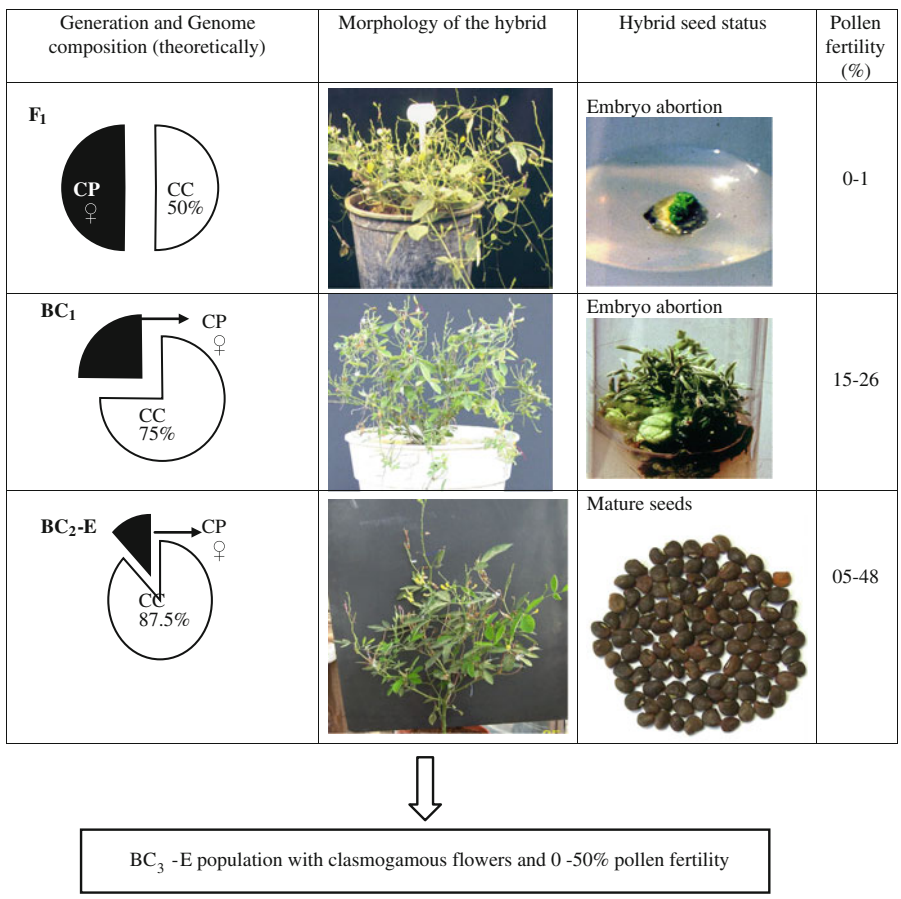

Fig. 1 Identification of CMS trait from *C. platycarpus*

Table 1 Number of pollen fertile and sterile plants obtained when BC_3-E15 was crossed with pigeonpea cultivars

Pollen sterility	ICP 88014 (%)	ICPL 85010 (%)	Asha (%)	ICP 1444 (%)	ICPW 68 (%)
0–20	00	00	00	00	00
21–40	35	00	20	22	00
41–60	17	00	24	29	00
61–80	30	00	27	24	77
81–90	7	20	30	25	23
91–100	11	80	00	00	00
Pod set (%)	19	7	21	15	1

observed that anther cavities lacked the line of dehiscence and had thick anther wall, which prevented the dehiscence of anthers and release of pollen grains (Fig. 3g–j). In fact, at the stage of pollen grain maturity, anther cavity developed multi-layers and a thickened cell wall (Fig. 3j). Since dehiscence did not take place pollen grains were not released from the anther sacs. Anther sacs were forcefully ruptured by squashing the anthers and self pollinations were carried out. In spite of self pollinations none of the plants set seeds.

Meiotic study of the progeny revealed that metaphase proceeds normally with the formation of either seven bivalents and two tetravalents (Fig. 2f) or with 11 bivalents. Anaphase proceeded metaphase with normal disjunction of chromosomes (Fig. 2g) resulting in tetrad formation (Fig. 2h). It was only after the formation of tetrads that many of the pollen grains lost their contents and turned sterile.

Crosses between BC_3-E15 and cultivar ICPL 87119 (Asha) gave rise to an average of 21% pod set. A total of 56 plants were grown. Pollen fertility in the plants ranged from 19 to 80% (Table 1). It was possible to get pods when F_1 plants were selfed. Crosses between BC_3-E15 and cultivar ICP 88014 gave rise to 19% pod set. Pollen fertility in the progeny plants ranged from 0 to 79%, and only one plant had 100% sterile grains. Crosses with ICP 1444 gave rise to only fertile plants and pollen fertility in the plants ranged from 18 to 77% (Table 1). Crosses with *C. platycarpus* ICPW 68 gave rise to 1.0% pod set. Pollen fertility in the F_1 hybrids ranged from 15 to 38%. Except for the progeny from the cross involving ICPL 85010, whose progeny had high pollen sterility (80–100%) with majority of the plants with 100% sterility, majority of the progeny obtained utilizing other cultivars had pollen high fertility.

Discussion

In plant sexual reproduction, control of water movement plays an important role. During development, for example, young anthers must first take up water for growth, but at later stages anthers and pollen dehydrate before dehiscence. It is known in literature that aquaporins and other proteins represent a class of proteins that mediates the movement of water over cellular membranes and PIP2 aquaporins are required for efficient anther dehiscence (Bots et al. 2005) and the process may not be a simple process of desiccation of the locule wall (Keijzer 1987, 1999). Disruption of this process causes the anthers not to dehisce and the process may be a chain of events causing male sterility as observed in pigeonpea.

Crosses with different cultivars of pigeonpea showed cultivar ICPL 85010 as a good source of maintainer of CMS, as all the plants from the cross E15 × ICPL 85010 gave rise to highly pollen sterile plants (Table 1). Cultivars ICPL 88014, ICP 1444 and ICP 87119 as pollen donors, were restorers of fertility with higher pollen fertility and pod set. Wild species accession ICPW 68 had higher number of male sterile plants compared to the three (except cv. ICPL 85010) cultivars. Although A_2 cytoplasm derived from *C. scarabaeoides* is a stable CMS source and shows significant heterosis and is stable across environments, fertility restoration is inconsistent. Hence not ready as a source of CMS for utilization (Saxena 2008). Hence identification of maintainers and restorers of CMS is important for successfully using the source to exploit heterosis in pigeonpea. In the present study most of the pigeonpea cultivars restored fertility.

Cajanus platycarpus is the only wild relative from the tertiary gene pool of pigeonpea which has been

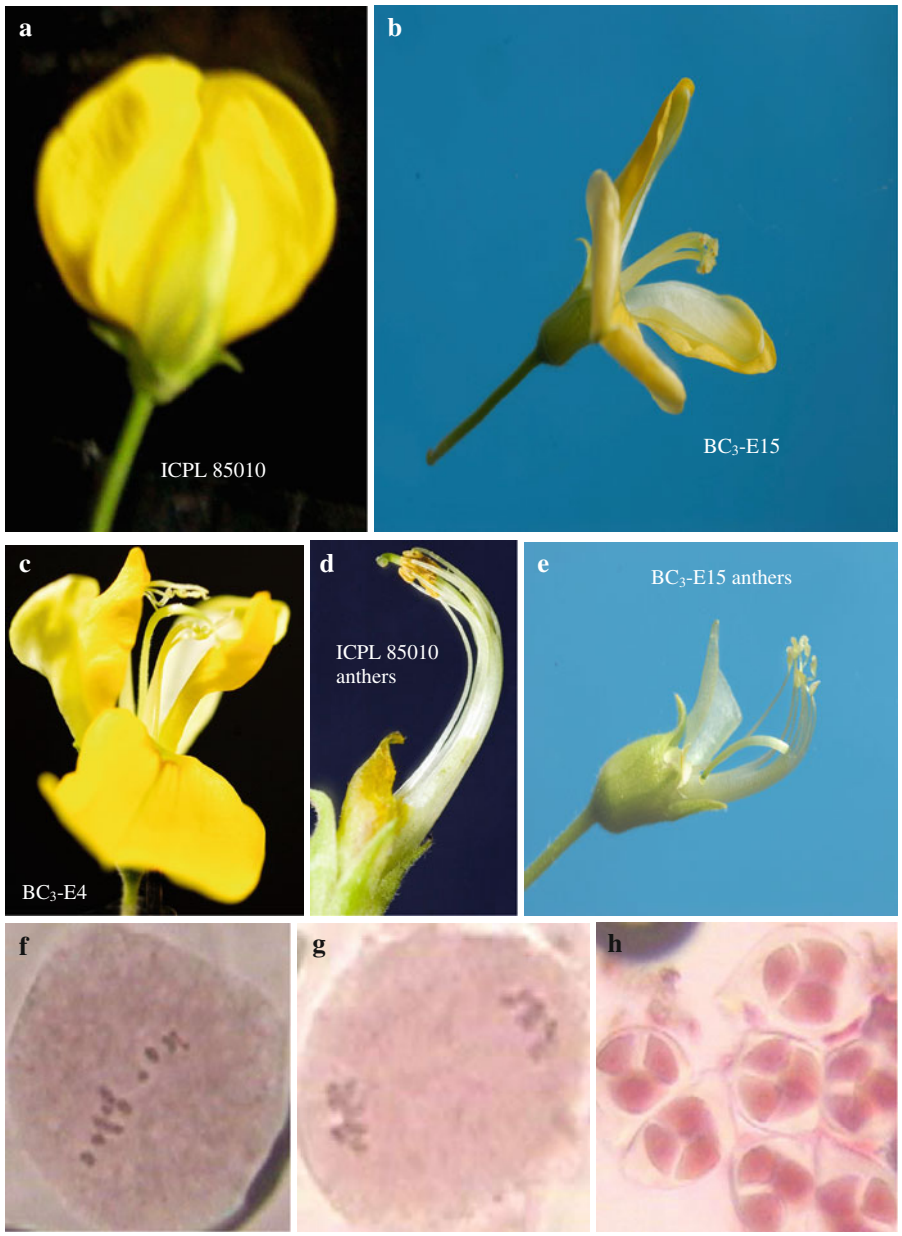

Fig. 2 Flower and anther morphology in ICPL 85010, BC_3-E4 and BC_3-E15 and cytology of BC_3-E15. **a** cv. ICPL 85010 flower. **b** BC_3-E15 open flower. **c** BC_3-E4 open flower. **d** Diadlephous yellow anther bundle of cv ICPL 85010. **e** Separated white anthers of BC_3-E15. **f** Metaphase of BC_3-E15, showing seven bivalents and two tetravalents. **g** Anaphase of BC_3-E15 showing normal separation of chromosomes. **h** Some tetrads seen in BC_3-E15

successfully crossed with the cultigen (Mallikarjuna et al. 2010). Screening advanced generation progeny for *Phytophthora* blight (PB) and *Helicoverpa armigera* (HA) has identified lines with low damage due to PB and low HA damage. Identification of CMS from *C. platycarpus* cytoplasm is another source for the exploitation of heterosis and diversification of the cytoplasmic base. It is envisaged that this system when fully developed should be able to effectively exploit heterosis as *C. platycarpus* is a tertiary gene pool species and its cytoplasm may be very diverse from that of cultivated pigeonpea.

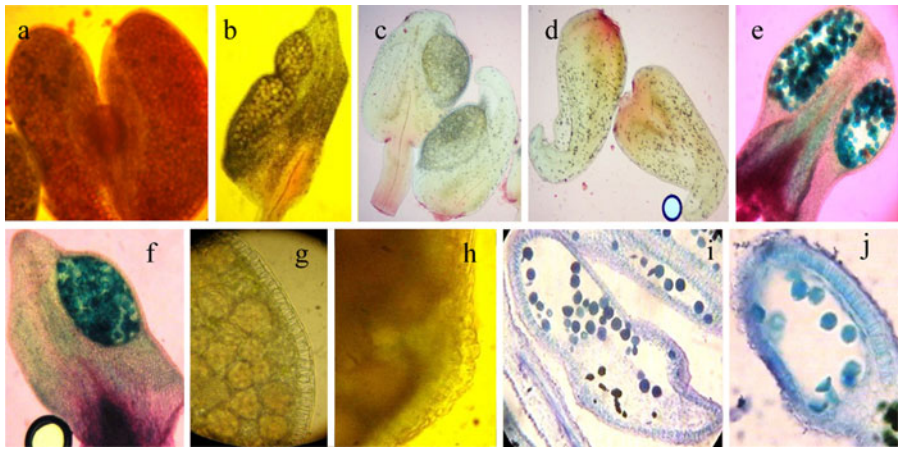

Fig. 3 Male sterile anthers in CMS A_7. **a** Anther of ICPL 85010. **b** Semi-sterile anther of E15. **c** Semi-sterile anther of E15. **d** Papery anther of E15. **e** Semi-sterile anther of E15 with Alexander's stain. **f** Semi-sterile anther of E15 with Alexander's stain. **g** Non-dehiscent anther. **h** Multilayers at the line of dehiscence. **i** Microtomy of the anther showing absence of line of dehiscence. **j** Anther cavity with thickened cell wall

The CMS source being reported is unique in having clasmogamous flowers, a trait favorable to develop CMS and exploit heterosis as it facilitates cross pollination. Coupled with clasmogamy, it was observed that the anthers were also free, again a trait favoring cross pollination. Superimposing these two traits, non-dehiscence of anthers is another trait favorable for the development of CMS. Open flowers and non-dehiscent anthers have not been observed in any of the already reported CMS systems. To conclude A_7 CMS source has a cascade of mechanisms to have non-viable pollen they are: premeiotic sterility, post meiotic sterility and open flowers with non-dehiscent anthers.

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