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Maternal inheritance of male sterility in the progeny of a natural hybrid between *Cajanus lineatus* and *C. cajan*

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Abstract

Adoption of pigeonpea hybrids in central and southern India is showing high impact with on-farm yield advantages of >30%. The hybrid pigeonpea technology, the first in any legume crop, is based on a cytoplasmic-nuclear male-sterility (CMS) system. For a long-term sustainability of hybrid programme, it is imperative that both nuclear diversity and cytoplasmic diversity are maintained among hybrid parents. In this context, a continuous search for new CMS-inducing cytoplasms is necessary. This paper reports detection of maternal inheritance of male sterility in the progeny derived from a natural hybrid between a wild relative [*Cajanus lineatus* (W. & A.) Maesen comb. nov.] of pigeonpea and an unknown pigeonpea [*Cajanus cajan* (L.) Millsp.] genotype. In the present study, the male sterility was maintained up to BC_7F_1 generation by an advanced breeding pigeonpea line ICPL 99044. This male sterility inducing cytoplasm of *C. lineatus* was tagged as A_6. In future, this CMS genetic stock can be used to develop a range of new pigeonpea hybrids with high yield and adaptation.

KEYWORDS
Cajanus cajan, *Cajanus lineatus*, male sterility, maternal inheritance, wild species

1 | INTRODUCTION

From plant breeding view point, the 20th century has a special significance because during this period not only the concept of heterosis evolved but was also commercially exploited for realizing quantum jumps in the productivity of a number of food crops. The key for this breakthrough was the success in breeding cytoplasmic-nuclear male-sterility (CMS) systems, which enabled large-scale seed production of hybrids with ease and at affordable costs. The hybrid technology has been highly successful in some cereals and horticultural crops but it was never considered for legumes because of their predominant self-pollinating nature. A breakthrough in this direction, however, was achieved when a stable CMS system was bred (Saxena, Kumar, Srivastava, & Shiying, 2005) in a partially cross-pollinated legume pigeonpea or red gram (*Cajanus cajan* [L.] Millspaugh). At present, three commercial pigeonpea hybrids are under cultivation in India; and these are based on a single (A_4) cytoplasm (Saxena, Sharma, & Vales, 2018). Such a cytoplasmic uniformity needs to be avoided in a long-term hybrid pigeonpea breeding programme. This is because if the cytoplasm carries extranuclear gene(s) that are susceptible to diseases and insect pests, then productivity of these hybrids may be adversely affected, as was experienced in the USA, when corn hybrids with T-cytoplasm became susceptible to leaf blight disease (Tatum, 1971). Hence, diversification of female hybrid parents with respect to their cytoplasm should be given due importance in a dynamic hybrid breeding programme. This paper reports a case of maternal inheritance of male sterility, which can be used to establish a new CMS system with the cytoplasm of a wild relative of pigeonpea (*C. lineatus* [W. & A.] Maesen comb. nov.).

2 | MATERIALS AND METHODS

Cajanus lineatus (W. & A.) Maesen comb. nov. (synonyms: *Atylosia lawii* Wight, *Atylosia lineata* Wight & Arn., *Cantharospermum lineatum*

[Wight & Arn.] Raizada) belongs to the secondary gene pool of genus *Cajanus* with somatic chromosome number similar to that of cultivated pigeonpea ($2x = 2n = 22$). In comparison with other *Cajanus* wild species, *C. lineatus* is considered to be genetically closer to the cultivated type, in fact only next to *C. cajanifolius* (Pundir & Singh, 1985), the wild progenitor of cultivated type (van der Maesen, 1980). Hence, it can be crossed easily with cultivated types.

Cajanus lineatus is a dicotyledonous perennial erect shrub with non-determinate growth habit (Figure 1). At flowering, the plants are 200–250 cm tall with open canopy and minute silky dense hairs all over. Its three leaflets are about 1.5 cm long and obovate with acute base. The petiole is 1–2 cm long, and calyx is acuminate. Flowers are yellow and 1–2 cm long and originate from axils. The ovary is densely villous. Pods are about 1–2 cm long with dense hairs; and on average, each contains 2–3 small greyish seeds with black mosaic. According to information available at the India Biodiversity Portal, *C. lineatus* is endemic to northern Sri Lanka and the Indian states of Kerala, Maharashtra, Karnataka and Tamil Nadu in India. It grows well in tropical dry and moist forests, under shady as well as sunny places, forests and hilly edges along roadsides. This species has been found growing between 400 and 1,600 m altitudes (van der Maesen, 1986).

In 2008 rainy season, an open-pollinated seed lot of *C. lineatus* was sown in the breeding block at ICRISAT, Patancheru. Due to hard seed coat, each seed was scarified with a sharp blade before sowing to ensure proper imbibition and germination. Sowing was done on a vertisol on ridges, spaced 75 cm apart with seed to seed spacing of 30 cm. The crop was raised with recommended cultural practices under irrigated conditions. Of 170 hills sown, 157 plants were obtained. One plant within this population appeared to be morphologically different from the rest (Figure 1).

At flowering, the odd plant was critically examined visually for various morphological traits such as plant vigour and height, flowering and branching pattern, and leaf type. These observations showed (although comparative data were not recoded) significant deviations from the *C. lineatus* plants and it appeared to be a natural hybrid between *C. lineatus* and an unknown pigeonpea genotype. As there was no pod set on this plant, its pollen viability was also studied. For this purpose, ten fully developed (but still closed) floral buds were harvested randomly from different parts of the plant and their anthers smashed on a glass slide and drenched in 2% aceto-carmine solution. Each slide preparation was examined under light microscope (10X), and the stained (fertile) pollen grains were counted. To protect the plant from physical damage and pollinating insects, it was covered with a 100 × 100 cm net made from a fine nylon mesh.

To maintain this unique genotype for further study, its eight pencil-thick stem cuttings were harvested, sterilized and planted in a 12″ pot filled with sterilized mixture containing four parts alfisols, two parts farm yard manure and one part sand. The pot was loosely covered with a polyethylene bag, and a Petri plate filled with water was kept inside the bag to maintain sufficient humidity and protect the cuttings from desiccation; and placed in a glass house. After three weeks, three cuttings started regenerating which ultimately grew into full plants. In the postrainy season, these plants flowered and were found to be similar to the mother plant with respect to their pollen viability.

In 2008 off-season, the vegetatively reproduced plants were crossed as female parents with an advanced breeding line ICPL 99044 [=ICPX 890059-SWB-SWB- SW5B-SWB] and 30 crossed seeds were harvested. These seeds were considered as BC_1F_1 [*i.e.*, F_1 (wild species × unknown pigeonpea plant) × ICPL 99044]. In the following rainy season, 21 BC_1F_1 plants were established through seeds. All the plants were carefully examined for their pollen viability; and those with less than 10% pollen viability were considered as male sterile; and among these, plants with least pollen fertility were selected for the next backcrossing with ICPL 99044. The pollinator plants were also selfed to develop the maintainer (B-) stock. By 2011, this material reached BC_4F_1 generation where four plants were found to be completely male sterile with shrivelled anthers. These were used for the next back crossing, and in BC_5F_1, only one progeny where all the plants were male sterile was selected for further backcrossing (Table 1).

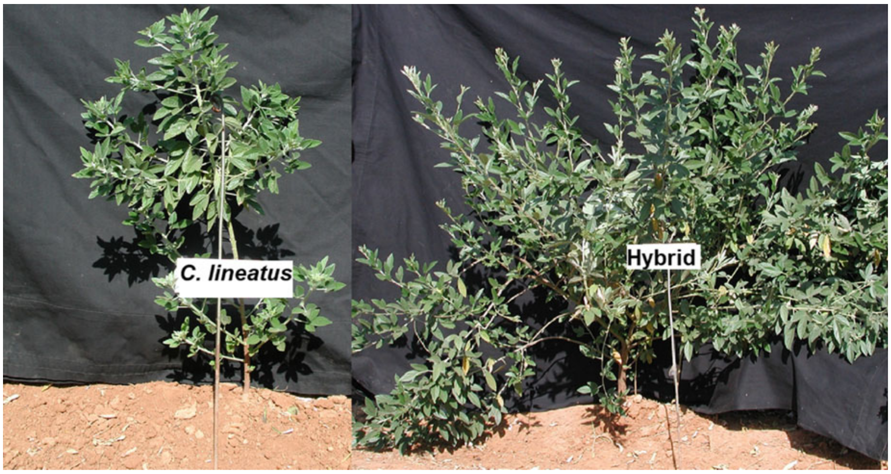

FIGURE 1 A comparison of *C. lineatus* a wild relative of pigeonpea (left) and its natural hybrid (right) identified in a pigeonpea field at ICRISAT

TABLE 1 Segregation for male sterility in different backcross generations

		Number of plants			
Generation	Year	Total	Fertile	Partial fertile	Male sterile
[a]F_1	2008	1	0	1	0
BC_1F_1	2008	21	0	16	05 (5–10)
BC_2F_1	2009	102	0	81	21 (5–10)
BC_3F_1	2010	97	0	66	31 (5–10)
BC_4F_1	2011	117	0	78	39 (0–10)
[b]BC_5F_1	2012	23	0	0	23 (0)
[b]BC_6F_1	2013	213	0	0	213
[b]BC_7F_1	2014	286	0	0	286

[a] Natural interspecific hybrid; () pollen fertility%

[b] The progeny of plant # 4 had 100% male sterility.

3 | RESULTS

The natural hybrid plant that was spotted within *C. lineatus* population was found to be partially male sterile; and in spite of 30%–50% pollen fertility in the flowers harvested from different parts of the plant, no pollen shedding and pod set was observed. To study the breeding behaviour of this variant, the vegetatively propagated plants were hand-pollinated using fresh pollen of a pigeonpea line ICPL 99044 and only 18% of the pollinated buds converted into pods. These observations suggested that the chance interspecific hybrid plant was male sterile but female fertile; and it appeared to be an ideal genetic material to explore the possibility of breeding a new male-sterility system.

Among the 21 BC_1F_1 plants grown, five exhibited ≤10% pollen fertility (Table 1) and the rest were partially fertile with 40%–70% pollen viability. In the next two backcross (BC_2F_1, BC_3F_1) generations also, the pollen viability of the plants was more or less similar and none of them was completely male sterile. However in BC_4F_1 generation, this scenario changed slightly where of the 117 plants grown, 78 were classified as partial fertile; and 39 plants with 0%–10% pollen fertility were considered male sterile. Among these four plants exhibited complete (100%) male sterility. Among the four BC_4F_1 progeny grown in 2011 rainy season, only one (#4) showed promise with the entire population being male sterile, suggesting that it was a case of a perfect cytoplasmic-nuclear male sterility (CMS). This hypothesis was further confirmed in the next two backcross generations (Table 1). This combination of the male sterile and its pollinator (=maintainer) lines was finally chosen for further seed multiplication of this new male-sterility system. Seeds of the male-sterile (A-) and its maintainer (B-) lines were, respectively, designated as ICPA 99044 and ICPB 99044 and the male-sterility system as A_6.

4 | DISCUSSION

Potential role of wild species in genetic enhance of cultivated types through creation of new variability is a well-recognized fact. The achievements of such endeavours may, however, differ from crop to crop; and in pigeonpea, it is limited to only a few successes (Mallikarjuna, Saxena, & Jadhav, 2011). In the present study, attempts were made to develop a male-sterility system from a natural hybrid plant identified in the open-pollinated population of *C. lineatus*, a wild relative of pigeonpea.

The natural hybrid plant was partially male sterile and did not produce any seed; hence, the F_2 generation could not be studied to understand breeding behaviour of the male-sterile trait and the backcross generations were relied upon for this purpose. In the initial backcross (Table 1) generations, none of the plants was fully male fertile and it suggested the absence of dominant fertility gene in its recurrent parent ICPL 99044. In fact, in each of the last three backcross (BC_5F_1 to BC_7F_1) generations, the inbred ICPL 99044 perfectly maintained male sterility and consequently, the entire progeny was male sterile. These observations showed that (i) selections made for male sterility in each backcross generation were effective in eliminating the factors that were imparting partial fertility to the plants, and (ii) the male sterility in *C. lineatus* was controlled by certain cytoplasmic factors and not by nuclear genes.

Expression of CMS in plant system is the consequence of certain deleterious interactions between its mitochondrial and nuclear genomes, which adversely affects the process of microsporogenesis (Kaul, 1988). Elkonin, Kozhemyakin, and Ishin (1998) observed that such interactions at the molecular level often induce changes at specific sites within mitochondrial genome. Hanson and Bentolila (2004) postulated that certain unusual open-reading frames (ORFs) which code for a polypeptide chain are responsible for the expression of CMS, while the male fertility is restored by specific nuclear genes, which encode through the production of pentatricopeptide. Chase (2007) and Sabar, de Paepe, and de Kouchkovsky (2000) attributed the formation of chimeric ORFs to certain recombination events in the mitochondrial genome; and these ORFs encode certain proteins, which result in reduced respiration and other mitochondrial defects leading to pollen sterility. In pigeonpea, Tuteja et al. (2013) reported that a total of 13 such ORFs differentiated the A_4 CMS line with its maintainer.

Reddy, Chandra, Pooni, and Bramel (2004) revealed that a considerable natural gene flow occurs within the genus *Cajanus* through insect-aided cross-pollination. In pigeonpea, the first reported on outcrossing was published by Howard, Howard, and Khan (1919); and it was followed by a number of similar reports from different countries (see review by Saxena, Tikle, Kumar, Choudhary, & Bahadur, 2016). No information on the cross-pollination in the wild relatives of pigeonpea was available until Tikka, Pawar and Chauhan (1997) reported the occurrence of a natural hybrid within the open-pollinated bulk of *C. scarabaeoides*, a wild species of secondary gene pool. Later, Saxena and Kumar (2010) reported natural cross-pollination in four wild relatives of pigeonpea belonging to the same gene pool. They recorded 17% natural outcrossing in *C. lineatus* and it was comparable to the control pigeonpea cultivar ‘Asha’ (21%). According to Saxena et al. (2016), such plant traits as pre-anthesis cleistogamy, extended stigma receptivity, nectar production and attractive large coloured petals were responsible for attracting the pollinating insects for foraging, which results in cross-pollination in the cultivated and wild relatives of pigeonpea.

Based on male sterility and natural outcrossing, the two natural systems, a commercially viable hybrid technology was developed (Saxena, 2015) and three commercial pigeonpea hybrids were released for cultivation in India. These hybrids are high yielding and widely adapted and farmers are harvesting 30%–50% more yield than the best inbred cultivars. In view of plateauing yield and potential of hybrids (Saxena, 2015), the hybrid breeding technology needs to be sustained through quality breeding programmes involving nuclear and cytoplasmic diverse parents.

Since at present the entire hybrid breeding programme is based on a single (A_4) cytoplasm (Saxena et al., 2018), the cytoplasmic uniformity may pose some problems in future with respect to productivity and adaptation of hybrids. Such a situation may arise if the mitochondrial genome of A_4 cytoplasm harbours gene(s) for any serious disease, insect or abiotic stress. In this context, the most striking example is that of corn in the USA, where once most commercial hybrids were based on a single (Texas) cytoplasm and it carried mitochondrial genes that were susceptible to Southern leaf blight (*Helminthosporium maydis*) disease. In 1969 and 1970 seasons, this disease appeared and spread across Corn Belt in the form of an epidemic and inflicted huge productivity losses (Hooker, Smith, Linn, & Beckett, 1970; Tatum, 1971; Ullstrup, 1972).

In spite of knowing the potential dangers, most commercial hybrid breeding programmes suffer from the weakness of cytoplasmic uniformity. For example, in rice Brar, Zhu, Ahmad, Jachuk, and Virmani (1998) revealed that so far more than 20 CMS systems have been reported, but 95% of the total hybrid rice area in China and elsewhere grows the hybrids based on a single CMS system, carrying the cytoplasm of *Oryza sativa f. spontanea*, popularly known as wild abortive (WA) cytoplasm. Besides this, there are other minor examples also, where certain extranuclear genes have shown harmful effects on the crop performance. In sorghum, for instance, A_1 cytoplasm was found susceptible to adult emergence of midge (Sharma, Vidyasagar, Abraham, & Nawanze, 1994) and shoot fly (B.V.S. Reddy pers. comm.). In pigeonpea also, A_4 cytoplasm has shown significant yield losses in certain hybrid combinations (Saxena, Sultana, & Rathore, 2015). The above examples show that although successes in diversifying the cytoplasm have been achieved, the commercial hybrid programmes are still short of cytoplasmic diversity.

In pigeonpea, the hybrid programme is very young and more breeding inputs are needed not only to expand the technology but to make it sustainable. The present study reports the identification of a new CMS system using the cytoplasm of *C. reticulatus*; and its A- and B-lines have been established. The immediate research priority is to stabilize and characterize both A-/B-lines for various agronomy traits and reaction to various biotic and abiotic stresses. Besides these, search for a stable fertility restoring system, diversification of parental lines and studies on heterosis and combining ability should be followed before a programme on the development and evaluation of hybrids is undertaken. This genetic material offers an opportunity to breed new hybrids carrying a different (*C. lineatus)* cytoplasm to benefit pigeonpea farmers of tropics and subtropics.

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