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Introgression of *Cajanus platycarpus* genome into cultivated pigeonpea, *C. cajan*

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Summary

Cajanus platycarpus, an incompatible wild species from the tertiary gene pool of pigeonpea (*C. cajan* (L.) Millspaugh), has many desirable characteristics for the improvement of cultivated varieties. To necessitate such transfers, embryo rescue techniques were used to obtain F_1 hybrids. The F_1 hybrids were treated with colchicine to obtain tetraploid hybrids, that were selfed to obtain F_2, F_3 and F_4 progenies. All of the hybrids and subsequent progenies had an intermediate morphology between the two parents. Backcrossing of the tetraploid hybrids with cultivated pigeonpea was not possible given embryo abortion, with smaller aborted embryos than those obtained in the F_0 parental cross.

As a route of introgression, diploid F_1 hybrids were backcrossed with cultivated pigeonpea and BC_1 progeny obtained by *in vitro* culture of aborting embryos. BC_2 plants were obtained by normal, mature seed germination. Although embryo rescue techniques had to be used to obtain F_1 and BC_1 plants, it was possible to produce BC_2 and subsequent generations through direct mature seed. Every backcross to cultivated pigeonpea increased pollen fertility and the formation of mature seeds.

Introduction

Wild species of crop plants are placed in secondary or tertiary gene pools based on their crossability with cultivated species. Pigeonpea (*Cajanus cajan* (L.) Millspaugh.) has a rich gene pool in its various wild species. Many of the wild species from the secondary gene pool are compatible with cultivated pigeonpea and have been successfully used to transfer genes and traits of interest (Saxena et al., 1992; Saxena et al., 2000; Saxena & Kumar, 2003; Mallikarjuna & Saxena, 2005).

There are also many wild relatives of pigeonpea that are incompatible with cultivated species but have desirable characteristics that would improve pigeonpea as a crop (Saxena et al., 1996; Reddy et al., 1996). Among these, *Cajanus platycarpus* has received considerable attention because it has many desirable traits important for pigeonpea improvement. Some of these traits are extra-early flowering and maturity, photoperiod insensitivity, prolific flowering and pod setting, annuality, rapid seedling growth, salinity tolerance, and resistance to phytophthora blight, cyst nematode, and *Helicoverpa* (Dundas, 1985; Subbarao, 1988; Reddy et al., 1996; Mallikarjuna & Moss, 1995). *Cajanus platycarpus,* although placed in the tertiary gene pool of pigeonpea, is now amenable to gene transfer with the development of suitable embryo rescue techniques (Mallikarjuna, 1998; 2003).

The major limitation in successfully using *C. platycarpus* for the improvement of cultivated pigeonpea is embryo abortion in the BC_1 generation from the cross *C. platycarpus* × *C. cajan*. Since embryo rescue is a time consuming technique in pigeonpea, our earlier attempts focused on treating diploid ($2n = 22$)

F_1 hybrids with colchicine to double the chromosome number and obtain tetraploid ($2n = 44$) F_1 hybrids (Mallikarjuna & Moss, 1995). The progeny were selfed to obtain tetraploid ($2n = 44$) F_2 plants. Selfing the F_2 plants gave rise to mature seeds. Reciprocal crosses using *C. cajan* as the female parent and *C. platycarpus* as the pollen donor were not successful.

Unfortunately, the selfed progeny (tetraploid, $2n = 44$, F_3 and F_4) from the cross *C. platycarpus* × *C. cajan* still had intermediate morphology with respect to growth habit, branching pattern, leaf and flower shape. In order to introduce more pigeonpea characters, F_1 (diploid, $2n = 22$) plants were backcrossed to cultivated pigeonpea.

The present study reports successful generation of backcross progeny by the use of *in vitro* techniques and conventional backcross program. The study also shows that it is possible to transfer important traits such as resistance to Phytophthora blight from *C. platycarpus*, although it is distantly related to cultivated pigeonpea.

Materials and methods

F_1 hybrid plants (*C. platycarpus* × *C. cajan*) were obtained by rescuing aborting hybrid embryos *in vitro* (Mallikarjuna, 1998). Apical buds of F_1 hybrids ($2n = 22$) were treated with an aqueous solution of 0.05% colchicine with 10% Tween-20 using a soaked cotton swab placed on the apical buds. After three days of colchicine treatment, apical buds were washed with water and allowed to grow. All the auxiliary buds and branches were excised. Hybrids were selfed to obtain tetraploid F_2 ($2n = 44$) progeny.

Embryo rescue and tissue culture techniques were as described by Mallikarjuna and Moss (1995). F_1 hybrids were backcrossed to the cultivated parent *C. cajan*. All the BC_1 ($2n = 22$) embryos aborted. To obtain BC_1 plants, aborting ovules/immature seeds were rescued using the technique developed to save F_1 hybrids. Aborting ovules/immature seeds from BC_1 plants ($2n = 22$) were supported on filter paper bridges and cultured on MS liquid medium with NAA (0.5 mg L^{-1}) and BAP (1.0 mg L^{-1}). After 3 weeks of ovule culture, embryos were dissected from the ovules and transferred to MS semi-solid medium with NAA (0.1 mg L^{-1}) and BAP (1.0 mg L^{-1}). Shoots which did not have a good root system were transferred to rooting medium which consisted of 1/10 MS basal medium with NAA (2 mg L^{-1}) and IBA (1.0 mg L^{-1}). After 15–18 days on the rooting medium, shoots were transferred to 1/10 MS basal medium devoid of any growth regulators. Shoots with robust root system were transferred to soil, grown and maintained in the glasshouse. BC_2 and BC_3 plants were obtained by mature seed germination.

The pathogen *Phytophthora drechsleri* Tucvker f. sp. *Cajani* was isolated from small pieces of 3 mm stem portions having lesions of Phytophthora fungi growing on pigeonpea plant. The stem pieces were washed in running tap water and surface sterilized in 2% sodium hypochlorite solution for 1–3 minutes and placed on potato dextrose agar (PDA) slants. On the basis of growth characteristics, slants with the fungus in pure form were identified and confirmed by microscopic examination. The P_3 isolate was confirmed by virulence test by inoculating 12–15 days old susceptible (ICPL 87119, susceptible to P_2 and P_3) and resistant seedlings (ICP 2366, susceptible to P_3 but resistant to P_2) with the inoculum. All the susceptible seedlings were killed by P_2 and P_3 isolates but among the resistant seedlings, they were healthy against P_2 isolate but succumbed to P_3 isolate.

F_2 seedlings with one trifoliate leaf ($\leq$15 days) were scored for the disease. The screening procedure was as follows: an inoculation concentration of 1g. of mycelium/100 ml of water, was sprayed on the seedlings. The seedlings were incubated at 25–30 °C at 95–100% humidity for 36 hours. Plants were sprayed with tap water every 2–3 hours during the day, until 4 days after inoculation. Disease data was taken after 10 days of inoculation. Plants which did not succumb to the disease were scored as resistant and the ones which succumbed to the disease were scored as susceptible. The screening procedure was as described by Gupta et al. (1, 8). After 30 days of sowing, the seedlings which did not succumb to the disease were again inoculated with Phytophthora pathogen and observations were recorded. Seedlings which showed resistance at the seedling stage were found to be resistant at 30 days too. Plants grew normally and set seeds.

Immature flower buds from F_1 diploid and F_1 tetraploid plants were fixed in Carnoy's II mixture (alcohol: acetic acid: chloroform; 6:3:1) at 4 °C for meiotic analysis. After 24 hours in Carnoys's II, buds were transferred to Carnoy's I (alcohol: acetic acid; 3:1). Buds were squashed and stained in 2% acetocarmine and meiotic analyses were made on suitable preparations. Pollen fertility analysis was conducted by staining the pollen grains in 2% aceto carmine solution. Pollen grains staining bright pink were counted as fertile grains and stainless grains were counted as sterile.

Chi – square (χ^2) analysis: Chi – square test (χ^2) to find the goodness of fit was calculated as per the formula using the genstat version 6.1(Payne, 2002).

$$\chi^2 = \Sigma \frac{(\text{Observed frequencies} - \text{Expected frequencies})^2}{\text{Expected frequencies}}$$

Results and discussion

Diploid F_1 hybrids from the cross *C. platycarpus* × *C. cajan* were obtained as a result of rescuing aborting hybrid embryos *in vitro* (Figure 1-6, 1-7, 1-8, 1-9). It took 6–8 months from embryo rescue to transfer of F_1 hybrid plants to soil. The F_1 hybrids had intermediate morphology, with semi-trailing branches. (Figure 4-2). The floral axis resembled that of *C. cajan*. In order to introduce more cultivated pigeonpea characteristics, F_1 hybrids were backcrossed with cultivated pigeonpea; however, embryos aborted in the BC_1 cross. In order to avoid embryo rescue for the second time, F_1 hybrids ($2n = 22$) were treated with colchicine to double their chromosome number. The success of doubling the chromosome number in the F_1 hybrids was 2%.

Tetraploid F_1 plants ($2n = 44$) had robust growth with intermediate morphology between the two parents with delicate branches and semi-trailing habit (Figure 1-3), and produced a large number of mature seeds. Tetraploid F_2 plants had robust growth with intermediate morphology between the two parents. There was not much difference in morphology between F_2 and F_3 hybrids (Figure 1-4, 1-5), and F_2 seeds were obtained in large numbers. The seeds had the shape of cultivated pigeonpea (Figure 3-5, 3-7) but with prominent strophiole and were black in color as in the wild species (Figure 3-6). In the F_3 ($2n = 44$), seeds were semi-black in color with a few tan colored seeds, and the size of the strophiole had reduced (Figure 3-8). In the field the tetraploid F3 plants because of their semi-trailing growth habit and large leaves covered the ground.

In order to check if genes from *C. platycarpus* introgressed into cultivated pigeonpea, a small random sample of 60 F_2 seeds were germinated in vivo and the seedlings were screened for Phytophthora blight disease, as *C. platycarpus* is the only wild species with reported resistance to the disease (Reddy at al., 1996). The progeny segregated into 1R:3S the expected Mendelian segregation ($\chi^2 = 0.02$; $p = 0.88$). A random sample of 46 F_2 derived F_3 seedlings were screened for Phytophthora blight, with 9 seedlings resistant and 37 seedlings susceptible. The Chi-square values fitted into the ration of 1:3 and was significant at 5% (<0.05). Selfing the plants to produce F_3 (Figure 3-8) and F_4 seeds were effortless and there was little change in plant and seed morphology. It was not possible to backcross any of the tetraploid progeny (F_1, F_2, F_3 or F_4) with cultivated pigeonpea to introduce more pigeonpea characteristics, as hybrid embryos were abnormal and aborted much earlier than in the F_0 cross. They did not respond to embryo rescue techniques due to their smaller size.

Table 1. Seed set in the cross *Cajanus platycarpus* ICPW 69 × *C. cajan* ICPL 85010.

	F_1	BC_1	BC_2	BC_3
No. pollinations	1183	788	901	506
No. pod set (%)	259 (22)	220 (28)	207 (23)	242 (48)
No. immature pods (%)	259 (22)	209 (27)	85 (9)	45 (9)
No. mature pods (%)	0	11 (1)	122 (14)	197 (39)
% pollen fertility	8	26	38	55

The diploid F_1 plants were backcrossed to cultivated parent. Embryos aborted between 15–20 days after pollination as seen in F_0 parental crosses, but the size of the aborting embryo was bigger than in the F_0 cross, being 0.25–0.5 mm in size, and at the cotyledonary stage of development. Embryos grew and formed plants on the embryo rescue medium developed for embryos from *C. platycarpus* × *C. cajan* cross.

The diploid BC_1 hybrid plant had intermediate growth habit with semi-trailing branches. Morphologically, there were no differences between F_1 and BC_1 plants. A small number (1%) of mature and a large number (27%) of immature seeds were obtained on the BC_1 plants (Table 1). Only the mature seeds were used to develop BC_2 plants.

BC_2 plants had more of cultivated pigeonpea characteristics, with erect growth habit (Figure 4-3). Flowers were tripped to encourage the development of seeds, but seed set from self pollinations was not observed in spite of increased pollen fertility of 38% (Table 1). BC_2 plants were backcrossed to cultivated pigeonpea and a large number of seeds were obtained. With the further introduction of the cultivated pigeonpea genome BC_3 diploid plants (Figure 4-4) resembled the cultivated parent that was also the pollen parent (Figure 4-5, 4-6).

F_1 hybrid pods ($2n = 22$) morphologically resembled the female parent *C. platycarpus* pods

Figure 1. (1) Female parent, wild species *Cajanus platycarpus*. (2) Male parent, cultivated pigeonpea *Cajanus cajan*. (3) F_1 (tetraploid) hybrid between *C. platycarpus* × *C. cajan*. (4) F_2 (tetraploid) hybrid between *C. platycarpus* × *C. cajan*. (5) F_3 (tetraploid) hybrid between *C. platycarpus* × *C. cajan*. (6) In-ovulo embryo culture to save aborting hybrid embryos. (7) Hybrid embryo culture. (8) Multiple shoots from hybrid embryo. (9) In vitro rooting of hybrid shoots.

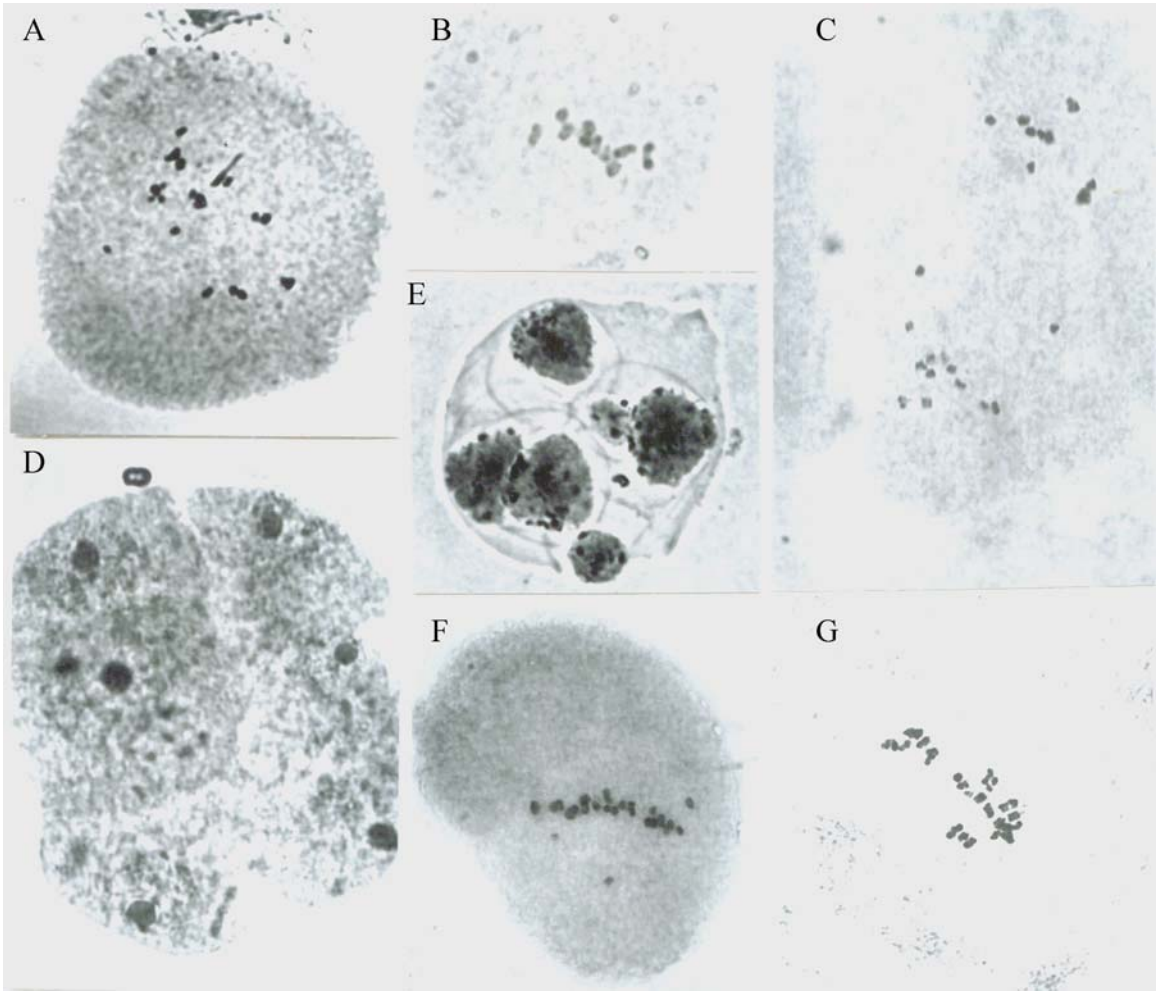

Figure 2. Meiotic analysis in the cross *C. platycarpus* × *C. cajan*: A–E F_1 diploid; A – F_1 hybrid showing 6 univalents. B – F_1 hybrid showing 12 bivalents, which is rarely seen. C – F_1 anaphase separation showing chromosomes not reaching the poles. D – F_1 telophase showing abnormal micronuclei in the tetrad. F – F_1 (tetraploid) metaphase showing the formation of univalents. G – F_2 (tetraploid) normal chromosome configuration in metaphase.

(Figure 3-1). F_2 pods resembled more the cultivated parent. Morphologically, there were no differences between F_2 (Figure 3-2) and F_3 pods (Figure 3-3). The BC_1, BC_2 and BC_3 pods resembled the female parent (Figure 3-4).

Meiotic analyses of the F_1 diploid hybrid showed much variation in chromosome configurations with a mean of 6 univalents (ranging from 5-8) and 8 bivalents ranging from 7-9, trivalents and tetravalents were not observed. F_1 tetraploid hybrid had predominantly bivalent formation ranging from 10–14, with a mean of 10 per cell and a mean of 6 tetravalents per cell. Univalents were rarely seen and trivalents were absent. BC_1 plants showed a 1.5 univalents per cell ranging from 1–2, with a mean of 9 bivalents, trivalents and tetravalents were rarely seen (Table 2; Figure 2).

Table 2. Chromosome association (per cell*) in F_1 diploid, F_1 tetraploid and BC_1 hybrids.

Hybrid	Univalents	Bivalents	Trivalents	Tetravalents
F_1 diploid	6.0	8.0	0.0	0.0
F_1 tetraploid	0.0	10	0.0	6.0
BC_1 diploid**	1.5	9	0.4	0.4

*mean of 50 cells; **mean of 44 cells.

The number of mature and immature seed set varied according to the generation, suggesting a relationship

Figure 3. (1) Pod morphology of the two parents (left- female parent; right – male parent) and the hybrid at the center. (2) F_2 (tetraploid) pods. (3) F_3 (tetraploid) pods. (4) F_1BC_2 (diploid) pods. (5) Seeds of male parent, the cultivated species *Cajanus cajan*. (6) Seeds of female parent, the wild species *Cajanus platycarpus*. (7) Tetraploid F_2 seeds. (8) Tetraploid F_3 seeds. (9) Diploid F_1BC_2 seeds.

to the amount of cultivated genome present. In the F_1 hybrid, which theoretically had 50% of wild species and 50% of cultivated genome, all pollinations had to be assisted by a growth regulator and all the seeds were immature. In the BC_1 hybrid with theoretically 75% of cultivated genome and 25% of the wild genome, there was improvement in pollen fertility from 8% in F_1 to 26%, but the number of immature seeds far exceeded the number of mature seeds (Table 1). In the BC_2, pollen fertility increased to 38% and more than 10% of the seeds were mature. It was not until the BC_3 generation that it was possible to obtain more than 35% mature seeds (Figure 3-9).

When using wild species from the tertiary gene pool, it is usually necessary to use embryo rescue techniques at least once to obtain hybrid plants. Such a requirement has been observed in groundnut and chickpea wide crosses (Mallikarjuna & Sastri, 2002, Mallikarjuna, 2003). In the present investigation, embryo rescue techniques had to be used more than once,

but by doing so it was possible to transfer more pigeonpea characters into the hybrid that were clearly evident in the resulting morphology. Theoretically in each backcross generation, 50% of the genome of the hybrid plant was replaced by the genome of cultivated species. It took three backcrosses (BC_3) to the cultivated pigeonpea to incorporate the morphology of cultivated pigeonpea in the hybrid plants.

This is the first report in pigeonpea where an incompatible wild species from tertiary gene pool such as *C. platycarpus* has been successfully crossed with cultivated pigeonpea and fertile hybrids and backcross progeny obtained. Efforts are being made to obtain seeds in large number so that the progeny can be used to screen for various biotic and abiotic constraints. In rice, resistance to brown plant-hopper, white-backed plant-hopper and bacterial blight was transferred from wild species using embryo rescue techniques thrice to obtain F_1, BC_1 and BC_2 hybrids (Brar & Khush, 1997). But the source of resistance transferred in 1978 to bacterial blight from the wild species is being used even today. Hopefully, with the success in pigeonpea × *C. platycarpus* crosses, similar transfers of enhanced resistance can be achieved.

The tetraploid hybrid plants form good ground cover, and hence can be used a forage plant and can be used as a graze for animals.

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