Tissue Culture Approaches to Pigeonpea Improvement

N.C. Subrahmanyam,[1] P. Sateesh Kumar,[2] and D.G. Faris[3]

Abstract

Pigeonpea anthers were cultured on different media in an effort to develop haploids. MS medium supplemented with 2 mg L^{-1} of 2,4 dichlorophenoxy acetic acid (2,4-D) supported the development of callus from Cajanus cajan *anthers, while potato starch extract medium promoted the best response from anthers of* Atylosia gradifolia *and* A. volubilis. *Such calli failed to differentiate on subculturing on media supplemented with combinations of hormones. Screening for chromosome elimination following intergeneric hybridizations also did not meet with any success in haploid formation. Culture conditions for plantlet regeneration from immature embryos of* Cajanus *have been standardized for further embryo rescue following distant hybridizations. An age-dependent embryo response was evident. Embryos older than 11 days developed into plants on MS or B5 media supplemented with 2,4-D (1 mg L^{-1}). The B5 medium was superior to MS for regeneration. Plantlets were also regenerated from explants of cotyledons from mature seeds, leaves and epicotyls of seedlings of* Cajanus *cultivars and* Atylosia *species in different media supplemented with hormones [2,4-D, naphthalene acetic acid (NAA), benzyl adenine (BA), kinetin and gibberelic acid (GA3)]. Whole cotyledons or their proximal segments were found to be suitable for regeneration.*

Introduction

Pigeonpea (*Cajanus cajan*) is an important pulse crop in the tropics, and ranks fifth, in area grown, among the edible legumes. Diverse gene pools have been the foundations for effective crop improvement programs. Among options open to plant breeders to widen the genetic base are exploitation of alien variation and somaclonal variation. There has been a rapid accumulation of literature on wide hybridization in crop plants (Collins and Grosser 1984). Pigeonpea has a wealth of related wild species in the genus *Atylosia*, with the same basic chromosome as pigeonpea (x=11). Many of the *Atylosia* species possess desirable characters such as disease and insect resistance, high seed protein content, photoperiod insensitivity, drought and salt tolerance, and frost tolerance (Remanandan 1981).

Wide hybridization is often hampered by postfertilization barriers such as failure of endosperm development and subsequent degeneration of hybrid embryos. In such cases, viable hybrids have been produced using embryo rescue techniques (Raghavan 1977, Stewart 1981). Several studies have shown that certain *Atylosia* species hybridize successfully with *Cajanus* while others fail to cross (Table 1). Apart from being a novel source of variability, wide hybridizations in barley proved to be a potential means of haploid production (Kasha and Kao 1970) via selective chromosome elimination (Subrahmanyam and Kasha 1973). Pigeonpea is a long-duration, photoperiod-sensitive plant and can normally be grown once a year. Development of haploids would, therefore, have immense value in pigeonpea breeding in reducing the time required for developing inbred lines. Unfortunately, haploid induction

1. Department of Botany, University of Poona, Pune, India, (originally School of Life Sciences, University of Hyderabad, Hyderabad).
2. Department of Agronomy, University of Illinois, Urbana, USA.
3. ICRISAT, Patancheru, Andhra Pradesh 502 324, India.

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Table 1. *Atylosia* species successfully hybridized with pigeonpea.

Species	Reference
A. albicans	Kumar et al. (1985a) Pundir and Singh (1983, 1985a,b)
A. acutifolia	Dundas (1984), Dundas et al. (1986)
A. cajanifolia	Tripathi et al. (1984), Kumar et al. (1985a,b), Pundir and Singh (1983, 1985a,b)
A. lanceolata	Kumar (1986)
A. latisepala	Kumar (1986)
A. lineata	Deodikar and Thakar (1956), Kumar et al. (1958), Kumar and Thombre (1958), Reddy (1981a), Reddy and De (1983)
A. pluriflora	Dundas (1984), Dundas et al. (1986)
A. reticulata	Dundas (1984), Dundas et al. (1986)
A. grandifolia	Kumar (1985)
A. sericea	Deodikar and Thakar (1956), Reddy (1981b), Kumar et al. (1985a)
A. scarabaeoides	Reddy (1981c), Pundir and Singh (1983, 1985a,b)
A. trinervia	Pundir and Singh (1983, 1985a,b)

techniques in legumes have not shown much promise (Bajaj et al. 1980). Tissue-culture techniques are emerging as a major supplement to conventional plant breeding procedures (Vasil et al. 1982) due to the increasing importance of clonal propagation (Murashige 1974), haploid production (Chu 1982), and production of disease-free plants (Gengenbach et al. 1977). Such studies are limited in pigeonpea (Kusumakanta and Padmanabhan 1964, Shamarao and Narayanaswamy 1975, Mehta and Mohanram 1981, Kumar et al. 1984). In the light of these considerations, investigations were undertaken:

1. to assess the crossability relationship between *Cajanus* and its wild relatives,
2. to develop methods to improve species crossability,
3. to screen for haploids following wide hybridizations, and
4. to develop in vitro regeneration techniques in pigeonpea.

Material and Methods

Emasculation, hybridization, and hormone treatments were done according to Kumar et al. (1985a). MS medium (Murashige and Skoog 1962); BP 5P medium (Gamborg et al. 1968), White's medium (White 1954) and potato starch extract medium (Anonymous 1976) were used as the basal media. Sucrose at 3% concentration was used as the carbon source, and pH was adjusted to 5.6–5.8. Media were solidified with 0.8% Difco® agar.

Seeds (for cotyledon and embryo cultures) and flower buds (for anther cultures) were sterilized for 15 min in 10% chlorox (C[illegible] USA), washed thrice with sterile water and retained in sterile water for 30 min, followed by drying prior to inoculation. For cotyledon cultures, the seed coat was removed and the seed was split so as to obtain two distal and two nodal halves of the cotyledons. All operations were conducted under asceptic conditions in a laminar flow hood. The cultures were maintained at a temperature of 25 ±2°C under cool flourescent light (6.8 W m^{-2}). Four *Cajanus* cultivars were used for standardizing the culture conditions for immature embryos. For anther cultures, in addition to *Cajanus*, *Atylosia albicans*, *A. grandifolia*, and *A. volubilis* were tested.

Results

Crossability

Eight species of *Atylosia* (*A. albicans*, *A. caja[illegible]plia*, *A. grandifolia*, *A. lanceolata*, *A. latisepala*, *A. lineata*, *A. scarabaeoides* and *A. sericea*) hybridized with *Cajanus*, while four species (*A. mollis*, *A. platycarpa*, *A. rugosa*, *A. volubilis*) failed to cross with *Cajanus*. The crossability between *Cajanus cajan* and *Atylosia* species varied with both the cultivars and the species used (Table 2). Reciprocals were attempted of the 24 successful combinations, but success was very low (Table 2). In the crosses involving *A. mollis* and *A. volubilis* and *Cajanus* cultivar Pant A2, pod development was normal but the seeds from such pods were shrivelled and nonviable.

2. Pod set (%) in crosses of *Atylosia* spp with different cultivars of *Cajanus cajan* (reciprocals in parenthesis).

Pollen parent	Cultivars			
	Pant A2	Baigani	ICP 7035	C 11
A. lineata	19.5	12.3	1.4	15.2
A. albicans	9.2(0.2)	7.2	1.1	5.1
A. sericea	2.4(0.2)	3.0	0.8	1.7
A. scarabaeoides	4.6	2.4	0.5	1.5
A. cajanifolia	2.7	3.1	0.6	2.1
A. grandifolia	1.7	-	-	3.1
A. latisepala	0.9	-	-	2.0
A. lanceolata	1.7	-	0	0.9

Effect of hormone treatments

cajan cv Pant A2 was the female parent in hone studies. The successful combinations exhibited a uniform response to hormone treatments. GA3 was found to be superior irrespective of the *Atylosia* species used as pollen parent and irrespective of the cross combination at concentrations up to 50 ppm to controls (Table 3). For instance, when *A. grandifolia* was the pollen parent, pod set was increased from 2% in the control to 14% in treatments with 50 ppm of GA3. The optimum concentration was found to be 40-50 ppm. Higher concentrations were detrimental with complete failure at 80 ppm or above. Treatments with GA3 + kinetin mixture did not improve pod set, and were detrimental at higher concentrations in crosses of *C. cajan* with *A. albicans, A. cajanifolia* or *A. sericea*, as male parents (Table 3). Treatments showed that GA3 alone, or in combination with kinetin, increased the pod length at physiological maturity to 7 cm as compared with 4.5-5.5 cm in the controls. The number of seeds per pod increased from 1.6-2.2 in the controls, to 3.5-4.0 when GA3 or GA3 + kinetin treatments were given. Hormone treatments did not influence seed size.,

Among unsuccessful combinations, bud drop commenced within 2 days after pollination. GA3 treatments prolonged ovary development and delayed bud drop for varying periods depending upon the cross combination and hormonal concentration. When either *A. platycarpa* or *A. volubilis* was the pollen parent, bud drop commenced 2 days after pollination in the control. GA3 prolonged this period to 5 days. When *A. mollis* was the pollen parent, bud drop was delayed by 3-4 days following GA3 treatment. Increase in ovule size was evident in cross combinations where bud drop was delayed. In all reciprocal crosses, there was variation in response to hormones. Crosses involving *A. sericea* and *A. grandifolia* did not respond to any of the treatments. In *A. cajanifolia*, GA3 delayed bud drop while a mixture of GA3 and kinetin or kinetin alone were ineffective. A similar trend was observed in unsuccessful crosses involving *Cajanus cajan* as female parent.

Screening for chromosome elimination

The F_1 hybrids in general tended to be intermediate in morphology between the parental species. *Atylosia* characters such as seed strophiole, seed mottling, pod hairiness, and persistance of petals were expressed in the F_1 generation. Leaf shape of hybrids between *Cajanus* and *A. albicans* was intermediate, with an obtuse tip in the initial stages of growth.

Table 3. Pod set (%) in *Cajanus cajan* cv Pant A2 × *Atylosia* spp crosses following hormone treatments.

Concentration (ppm)	Pollen parent											
	A. albicans			*A. cajanifolia*			*A. gradifolia*			*A. sericea*		
	GA3	KIN	GA3+KIN	GA3	KIN	GA3+KIN	GA3	KIN	GA3+KIN	GA3	KIN	GA3+KIN
Control	7	7	7	2	2	2	2	2	2	1	1	1
10	8	8	6	3	2	1	1	3	1	2	0	1
20	8	8	6	3	2	1	1	3	1	2	0	1
30	10	7	7	4	2	1	4	2	1	3	2	2
40	13	6	8	11	3	1	5	1	0	7	3	1
50	17	8	3	7	3	0	14	2	0	5	2	1
60	4	5	1	3	2	0	3	3	0	10	1	0
70	1	4	0	0	2	0	0	2	0	0	2	0
80	0	5	0	0	1	0	0	2	0	0	1	0

Leaves that developed after 100 days on some branches resembled those of the *Cajanus* parent (having an acute leaf tip). The texture and leaf surface revealed similarities between leaves of the *Cajanus* parent and those of the *Cajanus*-like leaves produced on the hybrid. Both exhibited long trichomes with uniform spread (Kumar 1985). Leaves of the *A. albicans* parent have a dense population of trichomes, whereas the initially developed, intermediate-type leaves had a sparse population of short trichomes. Floral initiation occurred only on branches that had developed *Cajanus*-like leaves. Meiotic investigations revealed the hybrid nature of these F_1 hybrids (Kumar et al. 1984).

Embryo Culture

Since most of the *Cajanus-Atylosia* crosses are successful only with *Cajanus* as the female parent, selfed embryos from *Cajanus* were used for standardizing the embryo culture technique. Response of embryos from four genotypes of pigeonpea was tested on MS and B5 media supplemented with 2,4-D (1 mg L^{-1}). An age-dependent response was evident (Table 4). Eleven to 14-day-old embryos developed callus from which plantlets were obtained at low frequency. When 15–19-day-old embryos were cultured, seedlings were recovered directly, with small amounts of callus at the base of each plantlet. Direct seedling recovery and occasional callus formation were obtained after culturing embryos older than 19 days. Embryos younger than 11 days failed to respond. Since post-pollination GA3 treatments delay bud/pod drop in intergeneric crosses, further refinements in the embryo culture techniques are likely to help in obtaining desirable *Cajanus* × *Atylosia* hybrids that have not yet been possible.

Anther Culture

Callus was obtained from the anthers of *C. cajan* and *A. albicans* on MS medium supplemented with 2 mg L^{-1} of 2,4-D. Potato starch extract medium promoted callus development from anthers of *A. grandifolia* and *A. volubilis*. Callusing was more profuse from anthers of *A. albicans* and *A. volubilis* than from the other species. Attempts to induce differentiation by subculturing the callus on basal media supplemented with various hormone combinations were not successful. On subculturing, the callus turned brown and degenerated. The use of activated charcoal (100 mg L^{-1}) and polyvinylpyrolidine (1g L^{-1}) in the medium did not improve callus survival.

Tissue Culture

Exploratory attempts to standardize cult
tions for regeneration of *Cajanus* anc
plants from cotyledons, mature seeds and leaf, and epicotyl segments from 1-week-old seedlings, resulted in different degrees of success. Cotyledon was found to be the best explant for regeneration. Four cultivars of *Cajanus* and one accession each of *A. cajanifolia*, *A. albicans*, and *A. sericea* were used. Three basal media (modified MS, B5, White's) supplemented with various hormones [2,4-D, indole acetic acid (IAA), NAA, BA, kinetin (KIN), and GA3] either alone or in combination were tested. Preliminary experiments revealed superiority of modified MS medium. Regeneration potential of whole cotyledons, and nodal and distal halves of cotyledons was studied.

The modified MS medium, supplemented with 2,4-D (2 mg L^{-}) induced copious amounts of healthy callus irrespective of the region of the cotyledon

Table 4. Percentage plantlet recovery from embryo cultures of pigeonpea on MS and B5 media supplemented (1 mg L^{-1}).

		Cajanus cajan cultivars			
Embryo age	Media	Pant A2	Prabhat	ICP 7035	C 11
<11 days	MS	0	0	0	0
	B5	0	0	0	0
11–14 days	MS	.11	8	10	7
	B5	17	7	19	13
15–19 days	MS	73	69	81	67
	B5	74	88	93	84
>19 days	MS	57	66	71	55
	B5	87	90	89	84

used. Whole cotyledons and nodal halves of cotyledons on modified MS medium supplemented with 2,4-D (0.5 mg L^{-1}) and BA (2 mg L^{-1}) developed multiple shoots (37) in addition to small amounts of callus in 2–46% of the cultures depending upon the cultivars (Tables 5,6). Among the *Atylosia* species, multiple shoots developed from 21% of *A. cajanifolia* cultures, but were rare in the cultures of *A. albicans* and *A. sericea*. In cultures of the distal segments of the cotyledons, only shoot bud-initiation was observed at low frequencies after profuse callusing. From the explants with multiple shoots, rooting was obtained on MS medium supplemented with NAA (2 mg L^{-1}) and BA (0.5 mg L^{-1}). Basal medium supplemented with 2,4-D (0.5 mg L^{-1}) and NAA (1 mg L^{-1}) also induced multiple shoots from whole cotyledons and nodal cotyledonary segments. With this hormone combination, both shoots and roots developed from 2–14% of whole cotyledon explants
ian and from about 2% of *A. cajanifolia*
and 6). A low frequency of plantlet regeneration was obtained from nodal halves of the *C. cajan* cotyledons. In the cultures of *A. albicans* and *A. sericea* only shoot regeneration was obtained. Of the four *Cajanus* cultivars tested, three showed varying levels of regeneration, while the fourth cultivar (GS4) failed to respond. The response of *A. cajanifolia* compared well with the response of *C. cajan* cultivars. In general, wherever there was multiple shoot formation, only one or two shoots developed fully, while the others remained suppressed.

The present study revealed that crossability with *Atylosia* is significantly influenced by the genotypes used as *Cajanus* parent, and that postpollination hormone applications improve crossability. In the unsuccessful combinations, postpollination hormone treatments delayed bud/pod drop. Anther culture studies were not successful beyond the stage of callus induction (Bajaj et al. 1980). Cultivar differences with respect to the percentage of regeneration indicate genotypic variation for this trait.

Table 6. Percentage regeneration from cotyledon cultures of different cultivars of *Cajanus cajan* and species of *Atylosia* on modified MS medium supplemented with 2,4-D (0.5 mg L^{-1}) and BA (2 mg L^{-1}).

Species/cultivars	Whole cotyledon	Cotyledon segments	
		Nodal	Distal
C. cajan cv ICP 4726	38	33	7
C. cajan cv ICP 7035	46	31	13
C. cajan cv Pant A2	27	29	6
C. cajan cv GS4	2	–	–
A. cajanifolia	21	14	10
A. albicans	19	16	–
A. sericea	11	10	–

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. Percentage plantlet and shoot regeneration from cotyledon cultures of different *Cajanus cajan* cultivars and of *Atylosia* on modified MS medium supplemented with 2,4-D (0.5 mg L^{-1}), BA(2 mg L^{-1}), and NAA (1 mg/L^{-1}).

Species/cultivar	Whole cotyledon		Cotyledon segments			
			Nodal		Distal	
	Shoots	Plantlets	Shoots	Plantlets	Shoots	Plantlets
C. cajan cv ICP 4726	32	8	31	3	17	–
C. cajan cv ICP 7035	39	14	22	2	13	–
C. cajan cv Pant A2	26	2	23	12	–	
C. cajan cv GS4	–	2	–	–	–	–
A. cajanifolia	29	2	25	1	8	–
A. albicans	21	–	9	–	–	–
A. sericea	14	–	11	–	–	–

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