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Development of *Cajanus platycarpus* × *Cajanus cajan* hybrids through embryo rescue

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Abstract

Crosses were made between pigeonpea [*Cajanus cajan* (L.) Millsp.) and its wild relative *C. platycarpus* to diversify the existing gene pool for the traits like earliness, photo-insensitivity etc. through introgression of genes from the later. Hybridization was attempted between *C. platycarpus* × *C. cajan* genotypes Pant A-3, UPAS-120, BR-183, BR-65, ICP-6344, Bahar, BS-7-14-3, BS-7-8-2, Pusa-9 and ICP 7119. Since, the pollen of *C. platycarpus* failed to germinate on the stigma of *C. cajan*, the former was only used as the female parent. The crosses were only successful in case of *C. cajan* genotype Bahar, UP AS-120, Pant A-3 and BR-65. The hybrid pod did not develop properly and the ovule development was also poor. In order to rescue the hybrid embryo, it was cultured at different stages of development on MS medium supplemented with various concentrations and combinations of phytohormones. The best medium to rescue the hybrids through embryo culture was MS+NAA (0.5 mg/l) + BAP (0.5 mg/l). Only the hybrids *C. platycarpus* × *C. cajan* var. Bahar and *A. platycarpus* var. Pant A-3 could survive through embryo culture. These hybrids were properly rooted and transferred to the pots for their confirmation of hybridity and further evaluation. Isozyme of peroxidase was used to confirm the hybridity.

Key words: Pigeonpea, wide hybridization, *Cajanus platycarpus*, embryo rescue, tissue culture

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is an important grain legume grown in India in about 3.6 million hectares, and constitute about 85% of the world production. In spite of all the efforts made over past few decades, the productivity has remained constant at its low level, may be due to lack of high yielding genotypes with stable performance over the environment. The low yield and stability are ascribed to non-availability of efficient short duration, photo insensitive, disease and pest resistant genotypes. Lacks of genetic variability for some important yield component, even in the world germplasm collection have been attributed to the limited success achieved so far. Introgression of desirable gene complex from wild species into cultivated species has been suggested to further diversify the existing genetic variability in pigeonpea.

Cajanus platycarpus, a wild relative of pigeonpea possesses several useful traits that can be utilized to enrich the existing genetic variability for earliness, photo-insensitivity, resistance to phytophthora, rapid seedling growth etc. [1]. Attempts have been made in past to obtain interspesific hybrid between *Cajanus platycarpus* and *Cajanus cajan* but the hybrids thus obtained were highly sterile [2]. Thus, for successful development of hybrids between *Cajanus platycarpus* and *Cajanus cajan*, further refinement of technique is needed. The present study reports the development of inter specific hybrids between *Cajanus platycarpus* and *Cajanus cajan* through embryo rescue.

Materials and methods

Seed of *Cajanus platycarpus* accession ICPW 68 and *C. cajan* var. Pant A-3, Bahar, UPAS-120, BR-183, BR-65, ICP-6344, obtained from different sources *viz.*, Genetic Resourse Program, ICRISAT and Pulses Improvement Project Tirhut College of Agriculture, Dholi were sown in the experimental area of the Department of Genetics, Rajendra Agricultural University, Pusa. When the plants reached the flowering stage, crosses were made between *C. platycarpus* and *C. cajan*. In all the crosses *Cajanus platycarpus* was used as female parent. Emasculation was immediately followed by pollination between 9.00 a.m. to 2.30 p.m. About 15-20 days after pollination, surviving pods were harvested and sterilized in 0.5% mercuric chloride solution for 5 minutes. MS medium supplemented with various combinations and concentration of Phytohormones were used to culture the ovule, embryo, Shoot apex and nodal stem and induction of roots.

Ovule were dissected out from hybrid pods and cultured on MS medium + NAA (0.5 mg/l) + BAP (0.5 mg/l) solidified with agar (0.8%). After 4 to 6 weeks, the embryos were dissected out from the surviving ovules and cultured on MS medium NAA (0.1 mg/l) + BAP (1.0 mg/l) solidified with agar (0.8%). observations were recorded on cultured hybrid embryo. Some hybrid pods were also allowed to develop on the plants in

the field for 20-25 days. Embryos were dissected out directly from these hybrid pods and cultured on MS medium NAA (0.1 mg/l) + BAP (1.0 mg/l) solidified with agar (0.8%).

Shoot apex and nodal stem obtained from cultured embryo were transferred for multiplication on 1/2 MS + IBA (0.1 mg/l), 1/2 MS + IBA (0.2 mg/l), 1/2 MS + NAA (0.1 mg/l) + IBA (0.05 mg/l), 1/10 MS medium + NAA (0.1 mg/l) + IBA (0.05 mg/l) and 1/10 MS medium + NAA (0.2 mg/l) + IBA (0.1 mg/l). The hybrid shoots were subsequently transferred on 1/2 MS + IBA (0.1 mg/l), 1/2 MS + IBA (0.2 mg/l), 1/2 MS + NAA (0.1 mg/l) + IBA (0.05mg/l), 1/10 MS medium + NAA (0.1 mg/l) + IBA (0.05mg/l) and 1/10 MS medium + NAA (0.2 mg/l) +IBA (0.1 mg/l) for induction of roots.

The *in vitro* developed plantlets were transferred to the sterilized sand compost mixture in the sterilized plastic pots. They were irrigated with 1/10 MS salt solution and covered with bell jar. The bell jars were removed for gradual increasing period of time to progressively acclimatize the hybrid plantlets to low humidity conditions prevailing in the field. Isozyme profile of peroxidase in the crude extract of leaf tissue obtained from hybrids and their parents was studied using horizontal starch gel electrophoresis on 12% starch gel according to the method suggested by Shields *et al.* [3] and the gels were stained according to Veech [4].

Results and discussion

In the present study, an attempt was made to develop inter-specific hybrids between *Cajanus platycarpus* and *Cajanus cajan* through embryo rescue. When *Cajanus cajan* was used as female parent the pollen of *Cajanus platycarpus* germinated but failed to reach the ovary, while the reciprocal cross with *C. platycarpus* as the female parent resulted in fertilization, seed and pod development. Thus, in all subsequent crosses *Cajanus platycarpus* was used as a female parent [2]. A total of 6473 crosses made between *C. platycarpus* and different varieties of *C. cajan* resulted into 72 pod formation. The frequency of hybrid pod formation was maximum with *C. cajan* var. Pant A-3 (1.504%), minimum with the *C. cajan* var. UPAS-120 (0.686%) and no pod formation with vars. BR-183 and ICP-6344 (Table 1). Thus, a genotypic difference among varieties of *C. cajan* for hybridization success was observed as found in crosses of *Cicer arietinum* genotypes with *C. pinnatifidum* [5].

Two approaches were followed to rescue the hybrid embryos. In one approach, the immature ovules were dissected out from the surviving hybrid pods of different crosses collected after 15-20 days of pollination and cultured on MS basal medium supplemented with NAA (0.5 mg/l) and BAP (0.5 mg/l). This medium has been used for the culture of immature ovules of *C. platycarpus* × *C. cajan* [2].

Altogether 50.62 percent of cultured ovules could survive. Surviving ovules gradually turned brown and yielded viable embryo. Rest of the ovules turned black and was devoid of viable embryo. Genotypic difference was observed with respect to survival of hybrid ovule. Crosses made with *Cajanus cajan* var. Bahar gave the best response followed by Pant A-3. No ovules could survive from crosses with the variety UPAS 120 and BR-65. Thus, the survival of the ovules in culture was also dependent on the variety of *C. cajan* used in hybridization with *C. platycarpus* as found with pod formation. Genotypic differences for survival of the cultured ovule and seedling emergence was also found in hybridization between *C. arietinum* and *C. pinnatifidum* [5]. After 5 weeks, hybrid embryos were dissected out from cultured ovule and cultured on MS basal medium supplemented with NAA (0.1 mg/l) and BAP (1.0 mg/l). In another approach, immature hybrid embryos were obtained directly from the surviving pods and cultured on MS basal medium supplemented with NAA (0.1 mg/l) and BAP (1.0 mg/l). Compared to the ovule culture medium, the medium for embryo culture has lower concentration of auxin (NAA) and higher concentration of cytokinin (BAP) to promote better development of shoot as well as to facilitate the differentiation of multiple shoots.

In direct embryo culture, response was observed only in *Cajanus platycarpus* × *C. cajan* var. Bahar that

Table. 1. Interspecific hybridization between *C. platycarpus* × *C. cajan*

Sl. No.	Cross combination	No. of pollinations	No. of pods	No. of ovule cultured	No. of embryo cultured	
					Direct	From rescued ovule
1.	*C. platycarpus* × *C. cajan* var. Pant A-3	1862	28	40	8	4
2.	*C. platycarpus* × *C. cajan* var. Bahar	2564	32	30	20	19
3.	*C. platycarpus* × *C. cajan* var. UPAS-120	1166	8	6	4	2
4.	*C. platycarpus* × *C. cajan* var. BR-183	146	0	0	0	0
5.	*C. platycarpus* × *C. cajan* var. BR-65	578	4	5	1	-
6.	*C. platycarpus* × *C. cajan* var. ICP 6344	157	0	0	0	0
	Total	6473	72	81	33	25

showed response in 65% cultures. On the other hand, when embryos were obtained from rescued ovules, 60% embryos from cross *Cajanus platycarpus* × *Cajanus cajan* var. Pant A-3 and 84.2% embryos from the cross *Cajanus platycarpus* × *C. cajan* var. Bahar showed the response. Thus like other tissue culture responses, the success of embryo culture was also dependent on the variety of *C. cajan* in hybridization with *C. platycarpus* and var. Bahar showing the best response. Genotype is the major factor influencing tissue culture responses. Even the nutritional component of the medium ultimately show their effect through genotypic factors.

The responding cultured hybrid embryos showed various types of responses *viz.*, callus formation, development of existing shoot, development of root and multiple shoot formation (Table 2). Compared to the only existing shoot development and multiple shoot formation by Mallikarjun and Moss [2] callus formation was observed generally from the embryonic axis and rarely from the cotyledons in majority of cultured hybrid embryos. The embryonic axis is composed of newly formed meristematic cells and has higher internal concentration of phytohormones, which make it more suitable for callus formation and other tissue culture responses. Genotypic difference was observed for callusing response. Hybrid embryo from the cross with cultivar Bahar showed callus formation in 86.21% cultures followed by cross with the cultivar Pant A-3 that showed callus formation in 83.33% cultures. The formation of callus under culture conditions is reflected in change in basic architectural pattern of tissue by cell division. Loss of certain cell type and development of new cell types with cells becoming metabolically more active are examples of such changes [6]. Callus induction depends on the plant genotype, the source and origin of the explant and the physiological state of the tissue at culture [7]. The callus was generally green white and compact with moderate to good growth (Fig. 1:a-c). No differentiation was observed from the callus.

Table 2. Response of rescued hybrid embryo on MS+NAA (0.1 mg/l) + BAP (1.0 mg/l)

Response	Hybrids	
	Atylosia platycarpus × *Cajanus cajan* var. Pant A-3	*Atylosia platycarpus* × *Cajanus cajan* var. Bahar
Callus formation:		
% culture sowing response	83.33	86.21
Nature	Compact	Compact
Colour	Green white	Green
Growth		
Shoot development:		
% Culture showing response	Nil	75.86
Growth (cm.)	-	(0.90±0.35)
Multiple shoot formation:		
% Culture showing response	33.33	17.24
No. of shoot/culture	14±5.66	7.2±1.92
Growth (cm.)	(059±0.12)	(1.54±0.57)
Root development:		
% Culture sowing response	Nil	41.38
No. of root/culture	-	1
Growth (cm.)	-	1.33±0.24

+ = Poor; ++ = Moderate; +++ = Good

Cultured embryos from the crosses with cultivar Pant A-3 neither show any growth of existing shoot nor development of root. However, the embryos from crosses with cultivar Bahar showed development of existing shoot in 75.86% of cultures. 41.38% cultured embryos from crosses with cultivar Bahar showed root development also (Table 2) (Fig. 1:d-e). Ovule and embryo culture has been used to obtain hybrids from interspecific incompatible pollination in chickpea and pigeonpea [2, 3].

Multiple shoot formation from the embryonic axis was also observed in few of the cultures from the crosses with cultivar Pant A-3 and Bahar. The frequency and number of such multiple shoots were higher in from the cross with cultivar Pant A-3 compared to those with cultivar Bahar. However, the growth of multiple shoot was better in case of embryos from the crosses with cultivar Bahar (Table 2). The embryo culture response depends upon the genotype of the parent from which the embryo has been taken and among the two parents the genotype of female parent affects the most [8].

Since the numbers of hybrid embryo formed from the interspecific crosses were very few, micropropagation was also done of the rescued hybrid after embryo culture. The shoot apices and nodal stems from the hybrid shoots were cultured on different media to obtain multiple copies of hybrid plantlets. For shoot apex culture, the best response was found on half MS medium supplemented with NAA (0.5 mg/l) and IBA (0.05 mg/l) followed by 1/10 MS medium supplemented with NAA (0.01 mg/1) and IBA (0.05 mg/l). On both these media, the existing shoot showed good growth and there were multiple shoot formations also (Table 3) (Fig. 1b). However for nodal stem culture, the best response was found on 1/10 MS medium supplemented with NAA (0.2 mg/l) and IBA (0.1 mg/l) (Fig. 7) followed by 1/10 MS medium supplemented with NAA (0.1 mg/l) and IBA (0.05 mg/l) (Table 4). Thus the number of hybrid shoots was increased.

Once large numbers of hybrid shoots were formed, experiments were done for root development and acclimatization of the hybrid plantlets. Hybrid shoot multiplied under *in vitro*, were transferred on five different

Table 3. Hybrid shoot multiplication through shoot apex culture in pigeonpea

Media	% response	Existing shoot growth (cm.)	No. of branches	Multiple shoot	
				% response	No.
1/2 MS+IBA (0.1 mg/l)	53.85	5.47±3.58	1	Nil	-
1/2 MS+IBA (0.2 mg/l)	20.00	0.75±0.35	1	Nil	-
1/2 MS+NAA (0.1 mg/l) + IBA (0.05 mg/l)	75.00	14.25±6.82	1.50±0.55	12.50	-
1/10 MS+NAA (0.1 mg/l) + IBA (0.5 mg/l)	41.67	16.30±6.38	1.40±0.55	16.67	9.00±1.41
1/10 MS+NAA (0.2 mg/l) + IBA (0.1 mg/l)	40.00	5.50±2.83	1	Nil	-

Table 4. Hybrid shoot multiplication through nodal stem culture in pigeonpea

Media	% response	Existing shoot growth (cm.)	No. of branches	Multiple shoot	
				% response	No.
1/2 MS+IBA (0.1 mg/l)	00.00	-	-	-	-
1/2 MS+IBA (0.2 mg/l)	00.00	-	-	-	-
1/2 MS+NAA (0.1 mg/l) + IBA (0.05 mg/l)	17.39	2.63±1.25	1	25.00	3
1/10 MS+NAA (0.1 mg/l) + IBA (0.5 mg/l)	47.83	4.33±1.89	1.45±0.93	-	-
1/10 MS+NAA (0.2 mg/l) + IBA (0.1 mg/l)	80.00	3.05±2.28	1	50.00	2

media having different strengths of MS basal salts supplemented with different concentrations and combinations of IBA and NAA to identify the best media combination for root development. 1/10 MS salt supplemented with NAA (0.2mg/l) and IBA (0.l mg/l) was adjudged the best medium for root formation. But for the number of primary roots, 1/10 MS medium supplemented with NAA (0.1 mg/l) and IBA (0.05 mg/l) gave the best response. The root growth was the best on the 1/2 medium supplemented with IBA (0.1 mg/l) (Table 5).

The *in vitro* developed hybrid plantlets were transferred to the sterilized sand compost mixture in the sterilized plastic pots (Fig. 1f). They were irrigated with the 1/10 MS salt solution. The plants were covered with beaker. The beakers were removed for gradual increasing period of time to progressively acclimatize the hybrid plantlets to low humidity conditions prevailing in the filed. About 80% plantlets survived in the pots up to 3 months. However, they failed to survive and develop in the field condition may be because of poor acclimatization or higher temperature in the filed.

Mallikarjuna and Moss [2] reported post-zygotic barriers to hybridization between *C. platycarpus* and *C. cajan* and produced hybrids between them through embryo rescue. The plantlets obtained in the present study, when evaluated for their morphological and biochemical traits showed *in vitro* flowering. The flowers were similar to *C. platycarpus* and sterile, while the leaf, stem and roots were intermediate between the *C. platycarpus* and *C. cajan* var. Bahar. Presence of a large number of laggards in hybrid between *C. platycarpus* × *C. cajan* reported earlier [2] has indicated non-homology between the two parental genomes. The high degree of pollen sterility in the hybrids between *C. platycarpus* × *C. cajan* in the present study is in agreement with earlier report [2] and may be attributed to non-homology between the two parental genomes. Chromosomal variability has been reported in tissue culture and regenerated plants of *Hordeum*. Larkin and Scowcroft [9] indicated that plantlets regenerated from callus of hybrid embryo show recombination not observed in direct hybrids and suggested a brief callus phase of hybrid embryo would be sufficient to enhance genetic recombination not observed indirect hybrids. The problem of high degree of pollen sterility in *C. platycarpus* and *C. cajan* hybrids can be overcome by inducing enhanced level of chromosomal exchange between both genome during in vitro culture. We have tried callus-mediated regeneration to overcome the problem but could not succeed to get regeneration. Further refinement in technique is required.

Table 5. Rooting of *in vitro* developed shoot in pigeonpea

Media	% response	Root growth			
		Primary		Secondary	
		Number	Length (cm.)	Number	Length (cm.)
1/2 MS+IBA (0.1 mg/l)	53.85	6.43±3.69	11.43±6.42	33.43±13.56	0.74±0.32
1/2 MS+IBA (0.2 mg/l)	15.38	7.75±4.57	3.38±2.29	-	-
1/2 MS+NAA (0.1 mg/l) + IBA (0.05 mg/l)	44.74	14.06±8.02	8.47±5.44	21.35±16.14	0.99±0.39
1/0 MS+NAA (0.1 mg/l) + IBA (0.5 mg/l)	37.84	30.07±15.16	5.01±3.06	25.71±13.77	1.24±0.49
1/10 MS+NAA (0.2 mg/l) + IBA (0.1 mg/l)	60.00	10.00±5.10	6.08±3.23	11.67±5.16	0.98±0.41

Fig. 1. Cultured hybrid embryo showing (a) shoot development and white green compact callus formation, (b) Multiple shoot and callus formation, (c) Shoot growth, branching and multiple shoot formation, (d) Development of axillary shoot and root from cultured nodal stem, (e) Development of shoot and root from hybrid shoot and (f) Pot transfer of *in vitro* developed hybrid plantlets.

The hybrid between *C. platycarpus* and *C. cajan* and the parents used in the hybridization programme when studied for the isozyme pattern of peroxidase exhibited co-dominant expression for both cathodal and anodal bands present in *C. platycarpus* and *C. cajan*. This indicates that peroxidase isozyme pattern can be used to identify the interspecific hybrids in *C. platycarpus* and *C. cajan* hybridization programme (Fig. 2).

Thus, ovule embryo rescue for incompatible cross combination have been resorted to secure gene combinations that were not available within the limit of the species [10]. The technique has been used to intrograte desirable genes from the wild species *Cajanus platycarpus* and the different genotypes of *Cajanus cajan*. From observation recorded in the present investigation, it is apparent that inter specific hybrid between *C. platycarpus* and *C. cajan* can be obtained through embryo/ovule culture. To overcome the high degree of sterility, efforts should be made to induce enhanced level of chromosomal exchange between both genomes through callus mediated regeneration.

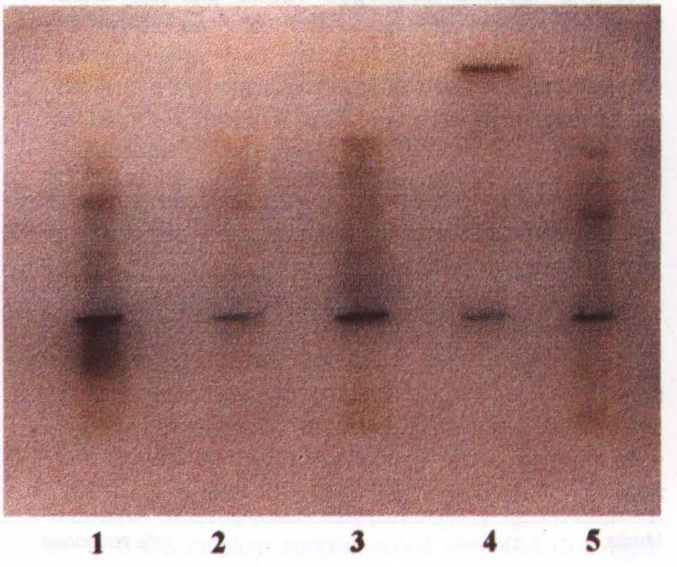

Fig. 2. Isozome pattern of peroxidase in *C. platycarpus*, *C. cajan* and their inter specific hybrids : Lane 1. *C. platycarpus* x *C. cajan*, Lane 2. *C. cajan*, Lane 3. *C. platycarpus* x *C. cajan*, Lane 4. *C. platycarpus*, Lane 5. *C. cajan*

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