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Rescue of *Cajanus cajan* (L) Millspaugh × *Cajanus platycarpus* (Benth.) van der Maesen Hybrid through Embryo Culture

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ABSTRACT: Genetic improvement of *Cajanus cajan* (L.) Millspaugh through gene introgression involving wild species of the genus using conventional methods is hampered due to the prevailing post-zygotic barriers. An *in vitro* technique has been used for rescuing the hybrid embryos between *C. cajan* and *C. platycarpus*, that are otherwise destined to abort under *in vivo* conditions. 11 to 15 days old embryos developed into only undifferentiated callus on both MS and B5 medium supplemented with 2,4-D/IAA alone and in combination with Kinetin. On the other hand, 16-20 days old embryos could be successfully rescued on MS/B5 + 1.0 mgL^{-1} IAA + 0.5 or 1.0 mgL^{-1} Kinetin. Successful embryo rescue operation is largely governed by age of the explant, apart from other decisive factors like hormonal combinations and their concentrations in the culture medium.

KEY WORDS : *Cajanus cajan*, *Cajanus platycarpus*, Immature embryos, Embryo rescue.

INTRODUCTION

Hybridization between closely related species is often hampered by post-fertilization failure of endosperm development and subsequent breakdown of developing embryos. The techniques of *in vitro* fertilization and embryo rescue methods (Stewart, 1981; Hu and Wang, 1986) have been extremely useful in circumventing the natural barriers of fertility. Distant hybridization programmes in several grain leguminous genera were largely benefited from embryo rescue and culture techniques (Braak and Kooistra, 1975; Bajaj *et al.*, 1982; Gosal and Bajaj, 1983; Ladizinski *et al.*, 1985). In the genus *Cajanus*, the prevailing crossability barriers between *Cajanus cajan* (L.) Millspaugh (pigeonpea) and some of the wild species warrants dependence on *in vitro* techniques for gene transfer through interspecific hybridization (Kumar, *et al.*, 1985).

Cajanus platycarpus (Benth.) van der Maesen, has been assigned to the tertiary gene pool of wild species which are not easily crossable with Pigeonpea (van der Maesen, 1990). However, this species is endowed with a number of desirable traits such as early flowering, high podset, high seed protein content, photoperiod insensitivity, prolific flowering and pod setting associated with high harvest index, annuality and rapid seedling growth (Dundas, 1985). Earlier workers did not succeed in obtaining hybrids involving *C. cajan* and *C. platycarpus* (Dundas, 1985; Ariyanayagam and Spence, 1978; Pundir and Singh, 1987). However, Mallikarjuna and Moss (1995) recorded hybrid between *C. platycarpus* and *C. cajan* by ovule culture and reported that the unrescued hybrid embryos were aborted 20 days after pollination.

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In the present study, an attempt to cross *C. cajan* with *C. platycarpus* by conventional hybridization procedures were unsuccessful owing to flower fall soon after pollination, apart from other post-zygotic barriers. The scarcely formed barren and underdeveloped pods containing small shrivelled masses in place of seed, indicated aborted embryos at different developmental stages. Hence, attempts were made to arrest abscission of cross pollinated flowers through application of the hormonal mixture (Indoleaceticacid (IAA) + Kinetin (KN) + Gibberellic acid (GA); 40 : 20 : 10 mgL^{-1}) to the pedicels upto 20 days and culturing the embryos at different developmental stages on suitable media. Results of *in vitro* experiments for rescuing the embryos that are otherwise destined to abort under field conditions, and the details relating to the nature of embryo response, precise culture conditions and media composition promoting embryo culture forms text of the present investigation.

MATERIALS AND METHODS

Developing pods containing 6 to 20 days old embryos of *C.cajan* (cv. ICPL 93115) obtained by self pollination and those of containing hybrid embryos of *C.cajan* and *C .platycarpus* cross, were collected from the experimental field. Microscopic examination of the 11 to 20 days old excised embryos were found to be in heart shape to early cotyledonary stage with shoot apex and cotyledons, while those beyond the age of 10 days were not amenable for manual dissection.

MS (Murashige and Skoog, 1962) and B5 (Gamborg *et al.*, 1968) media supplemented with 2,4-D/IAA; 2,4-D/IAA + KN (Table 1) were used for embryo culture. The field collected hybrid pods were surface sterilized twice with alcohol and 0.1% $HgCl_2$ for about 5-6 minutes and thoroughly washed with sterilized water before dissecting the embryos. The cultures were maintained at 23 ± 2℃ and 10/14 hours of light/dark periods.

RESULTS AND DISCUSSION

Callus was initiated from the explants in about 7-10 days after inoculation and by the 15th day the dissected embryos increased 2-3 times the original size on MS as well as on B5 media and the response was highly variable among the of different ages (Table 1). Ten days old cultures on both the media supplemented with hormones produced callus in more than 50% of the 11 to 15 days old embryo explants while the rest did not show response. About 70-90% of the responding embryos of 16-20 days old developed into seedlings on MS/B5 + IAA 1.0 mgL^{-1} + KN 0.5/1.0 mgL^{-1} (Tables 2 and 3, Fig. 5). On the other hand, about 10 to 30% of 16 to 20 days old embryos showed either undifferentiated callus or differentiated into shoot alone (Fig. 3) or shoot and feeble roots (Fig. 4).

Effect of 2, 4-D concentration on the quantity of callus produced was assayed by visual examination of 15 days old cultures. Increase in callus production was recorded with increase in concentration of 2, 4-D. The morphology of callus obtained on MS/B5 medium with 2, 4-D and KN was friable, pearly white and glistening while on MS + IAA + KN, it was compact, nodulated and chalky white. The friable callus was soft and amorphous while the nodulated callus was very hard and compact. In both the calli, no morphogenesis or embryogenesis was observed upto three subcultures either on MS/B5 basal media or the same media used for culturing the embryos 〔 MS/B5+IAA (1.0 mgL^{-1}) + KN (0.5 mgL^{-1}) 〕.

Table 1. Effect of growth regulators on immature embryos of pigeonpea cultured on MS and B5 media*.

Medium	PGRs concs. (mgL^{-1})			Age@ of the explant and its nature of response																
				11-15	16			17			18			19			20				
				C	C	Sh	R	C	Sh	R	C	Sh	R	C	Sh	R	C	Sh	R		
MS	2,4-D	+	KN																	
	0.0	+	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	0.5	+	0.0	+	+	+	-	+	+	-	+	-	-	+	+	-	+	++	-		
	1.0	+	0.0	+	++	+	-	++	+	-	++	+	-	++	+	-	+	++	-		
	1.0	+	0.5	+	++	+	-	++	+	-	++	+	-	++	+	-	++	++	-		
	1.0	+	1.0	+	+	+	-	+	+	-	++	+	-	++	+	-	+	+	-		
	IAA	+	KN																		
	0.5	+	0.0	+	+	+	-	+	+	-	+	+	+	+	+	-	+	+	+		
	1.0	+	0.0	+	+	++	-	+	+	-	+	+	+	+	++	+	+	++	++		
	1.0	+	0.5	+	++	+	-	++	++	-	+	+	+	+	++	++	-	++	++		
	1.0	+	1.0	+	+	+	-	+	+	-	+	+	+	+	++	+	-	++	++		
B5	2,4-D	+	KN																		
	0.0	+	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	0.5	+	0.0	+	-	-	-	+	+	-	+	-	-	++	+	-	+	++	-		
	1.0	+	0.0	++	++	+	-	++	+	-	++	+	-	++	+	-	++	++	-		
	1.0	+	0.5	+	+	+	-	++	+	-	++	+	-	++	+	-	+	++	-		
	1.0	+	1.0	+	+	+	-	+	+	-	++	+	-	++	+	-	+	+	-		
	IAA	+	KN																		
	0.5	+	0.0	+	+	-	-	+	+	-	+	+	+	+	+	-	+	+	+		
	1.0	+	0.0	+	++	+	-	+	+	-	+	+	+	+	++	+	+	++	++		
	1.0	+	0.5	+	++	++	-	++	++	-	+	++	+	+	+	++	-	++	++		
	1.0	+	1.0	+	+	+	-	+	+	-	+	+	+	+	++	+	-	++	++		

*Response after 30 days of inoculation; @Days after pollination; PGRs = Plant growth regulators; C = callus; Sh = shoot; R = root;
' - ' = No response; + = Poor growth ; ++ = Better growth.

Table 2. Morphogenic response of immature embryos of pigeonpea cultured on MS and B5 media supplemented with IAA + KN[@].

Age of the explant*	PGRs concs. (mgL^{-1})	Embryos produced callus only		Embryos produced shoots only		Embryos produced plantlet[+]		Total embryos [++] responded	
	IAA+KN	MS	B5	MS	B5	MS	B5	MS	B5
11-15	0.0 + 0.0	-	-	-	-	-	-	-	-
	0.5 + 0.0	56.4 ± 1.21	60.0± 2.59	-	-	-	-	56.4 ± 1.21	60.0 ± 2.59
	1.0 + 0.0	62.6 ± 1.75	60.8 ± 2.75	-	-	-	-	62.6 ± 1.75	60.8 ± 2.75
	1.0 + 0.5	68.2 ± 1.99	66.2 ± 1.72	-	-	-	-	68.2 ± 1.99	66.2 ± 1.72
	1.0 + 1.0	60.0 ± 2.81	64.2 ± 1.96	-	-	-	-	60.0 ± 2.81	64.2 ± 1.96
16	0.0 + 0.0	-	-	-	-	-	-	-	-
	0.5 + 0.0	62.2 ± 2.20	68.6 ± 2.54	-	-	-	-	62.2 ± 2.20	68.6 ± 2.54
	1.0 + 0.0	64.0 ± 1.52	66.2 ± 2.15	-	-	-	-	64.0 ± 1.52	66.2 ± 2.15
	1.0 + 0.5	62.4 ± 1.91	64.0 ± 1.30	2.2 ± 037	4.0 ± 0.71	-	-	64.4 ± 2.28	68.0 ± 2.01
	1.0 + 1.0	60.2 ± 1.72	62.4 ± 1.91	6.4 ± 0.51	6.2 ± 0.66	-	-	66.6 ± 2.23	68.6 ± 2.57
17	0.0 + 0.0	-	-	-	-	-	-	-	-
	0.5 + 0.0	64.0 ± 1.30	62.0 ± 1.64	-	-	-	-	64.0 ± 1.30	62.0 ± 1.64
	1.0 + 0.0	68.2 ± 2.08	66.2 ± 1.88	-	-	-	-	68.2 ± 2.08	66.2 ± 1.88
	1.0 + 0.5	60.2 ± 2.04	62.4 ±1.57	6.4 ± 0.51	4.2 ± 0.37	-	-	66.6 ± 2.55	66.6 ± 1.94
	1.0 + 1.0	54.0 ± 1.64	62.0 ± 2.74	6.0 ± 0.32	6.2 ± 0.58	-	-	60.0 ± 1.96	68.2 ± 3.32
18	0.0 + 0.0	-	-	-	-	-	-	-	-
	0.5 + 0.0	64.2 ± 2.18	66.4 ± 1.44	4.0 ± 0.71	4.2 ± 0.37	2.2 ± 0.58	4.0 ± 0.63	70.8 ± 3.47	74.6 ± 2.44
	1.0 + 0.0	58.4 ± 2.40	60.0 ± 2.65	8.2 ± 0.86	6.0 ± 0.84	6.0 ± 0.71	10.0 ± 1.00	72.6 ± 3.97	76.2 ± 4.49
	1.0 + 0.5	48.2 ± 2.29	43.2 ± 2.11	9.4 ± 0.75	11.2 ± 0.66	23.2 ± 1.39	24.2 ± 1.77	80.8 ± 4.43	78.6 ± 4.54
	1.0 + 1.0	30.0 ± 2.70	33.0 ± 1.64	18.0 ± 0.55	14.2 ± 1.20	30.4 ± 1.44	27.2 ± 1.50	78.4 ± 4.69	74.4 ± 4.34

Table 2. continued.

Age of the explant*	PGRs concs. (mgL^{-1})	Embryos produced callus only		Embryos produced shoots only		Embryos produced plantlet[+]		Total embryos [++] responded	
	IAA+KN	MS	B5	MS	B5	MS	B5	MS	B5
19	0.0 + 0.0	-	-	-	-	-	-	-	-
	0.5 + 0.0	65.2 ± 2.01	66.0 ± 2.35	5.2 ± 0.86	7.4 ± 1.21	4.2 ± 0.86	3.2 ± 0.37	74.6 ± 3.73	76.6 ± 3.93
	1.0 + 0.0	60.0 ± 2.92	60.6 ± 2.97	10.2 ± 0.58	8.0 ± 1.00	8.6 ± 0.74	8.2 ± 0.86	78.8 ± 4.24	76.8 ± 4.83
	1.0 + 0.5	41.2 ± 2.76	46.2 ± 2.42	8.0 ± 0.84	9.2 ± 1.16	31.2 ± 1.07	29.0 ± 0.55	80.4 ± 4.67	84.4 ± 4.13
	1.0 + 1.0	36.0 ± 2.51	35.4 ± 2.54	4.2 ± 0.37	2.4 ± 0.51	44.0 ± 1.52	39.0 ± 1.41	84.2 ± 4.40	76.8 ± 4.46
20	0.0 + 0.0	-	-	-	-	-	-	-	-
	0.5 + 0.0	68.0 ± 2.70	64.2 ± 2.38	2.4 ± 0.25	1.0 ± 0.32	8.2 ± 1.28	11.2 ± 0.86	78.6 ± 4.23	76.4 ± 3.56
	1.0 + 0.0	70.2 ± 2.27	63.4 ± 2.34	-	-	10.4 ± 0.98	15.4 ± 0.81	80.6 ± 3.25	78.8 ± 3.15
	1.0 + 0.5	48.0 ± 2.72	45.2 ± 1.28	-	-	38.0 ± 1.64	37.2 ± 1.16	86.0 ± 4.36	82.4 ± 2.44
	1.0 + 1.0	30.4 ± 2.79	28.0 ± 1.48	-	-	54.0 ± 1.87	52.0 ± 1.58	84.4 ± 4.66	80.0 ± 3.06

[@]Number of explants cultured for each concentration in both MS and B5 media is 100 for each set
*Days after pollination; PGRs = Plant Growth Regulators; '-' = Swelling;
+ = Dominant seedling growth with incipient callus; ++ = Response on 30th day after inoculation
± values indicate standard error

Initiation of both shoot and root from 16 to 20 day old embryos from their primordia was possible in more or less in all the hormonal combinations tried, except MS/B5 + 2,4-D (0.5/1.0 mgL^{-1}) where the embryos showed strong tendency towards callusing. MS/B5 + IAA at different concentrations appeared to promote healthy growth of the embryos, rather than that of MS+2,4-D. Irrespective of the medium (MS or B5) rate of growth appeared to be governed by IAA quantities. Further, least callusing trend was apparent in the vertically placed explants on the medium.

Based on the technique standardized for rescuing selfed embryos of ICPL 93115 variety of *C. cajan*, the immature hybrid embryos between *C. cajan* and *C. platycarpus* were cultured on MS + IAA (1.0 mgL^{-1}) + KN (0.5 mgL^{-1}). Successful plantlet development could be obtained from 16-20 days old embryos (Table 3, Figs. 5 & 6).

Advances in embryo culture methods have served to open the way to obtain plants effectively from inviable hybrids and to overcome different types of dormancies. In the present case, this method has been adopted as a pre-requisite for obtaining a system required for rescue of immature hybrid embryos (between Pigeonpea and its wild species) and to overcome some post-fertilization barriers. Post-pollination hormone treatments in Pigeonpea interspecific crosses showed that hormone applications delayed pod drop as much as 8 days after pollination in some cross combinations (Kumar *et al.*, 1985; Dhanju *et al.*, 1985). But how early the young embryos could be dissected out and cultured will depend on the refinement of the technique. In the present study, the cross pollinated flowers could be retained intact till 20 days by hormonal application. Immature embryos from 11^{th} day onwards were excised from developing pods and cultured on MS and B5 media supplemented with different hormones. Their response clearly showed that embryos of 11 to 15 days old produced only callus irrespective of the type of growth regulators used. While 16 to 20 days old embryos responded differently with different growth regulators.

In general, no significant difference was observed with respect to growth response of the embryos of different ages cultured in the two media (MS and B5) (Table 2, Figs. 1 & 2). B5 medium was superior to MS for plantlet regeneration from 11 to 19 days old immature embryos (Kumar and Subrahmanyam, 1985) of pigeonpea. Difference in the genotypes of the cultivars used in the present study may probably account for the disparity. Non-embryogenic, friable and compact calli were obtained from 11 to 15 days old immature embryos which could not be regenerated into plantlets. It was observed in the present study that the embryos placed at a depth of about 4 to 5 mm below the agar surface did not grow well. Similar observations were made (Sen and Mukhopadhyay, 1961) while culturing embryo axes of mature seeds of some pulses (broad bean, horse gram, arhar and gram).

2,4-D checked the growth of seedling right after initial stage and showed a strong tendency towards callusing whereas presence of IAA encouraged seedling growth with scanty callusing. IAA alone or in combination with KN resulted in both seedling growth and callus formation. IAA of 1.0 mgL^{-1} +KN of 0.5 or 1.0 mgL^{-1} seemed to be promoting growth of the immature embryos in the present study. For culturing 11 to 19 days old immature selfed embryos of pigeonpea, 1.0 mgL^{-1} 2,4-D was found to be optimum (Kumar and Subrahmanyam, 1985) while B5 + IAA (4.0 mgL^{-1}) + KN (2.0 mgL^{-1}) to be suitable for embryo culture for *Arachis* (Bajaj *et al.*, 1982). But such requirements seemed to be species dependent. It is apparent from the present studies that successful embryo rescue operation is largely governed by age of the explant, apart from other decisive factors like type of hormonal combination and their concentration. This embryo rescue technique could be used to produce interspecific hybrids for use in the genetic transformation programme.

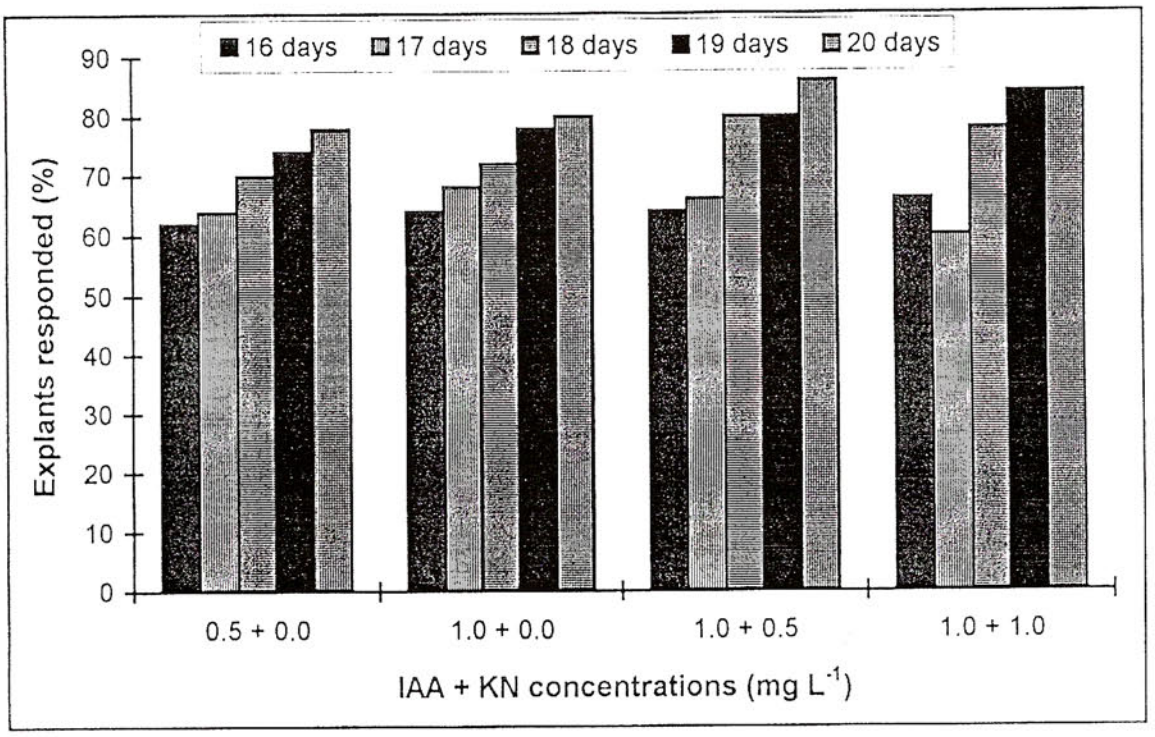


Fig. 1: Morphogenic response of Pigeonpea immature embryos of 16 to 20 days old cultured on MS medium.

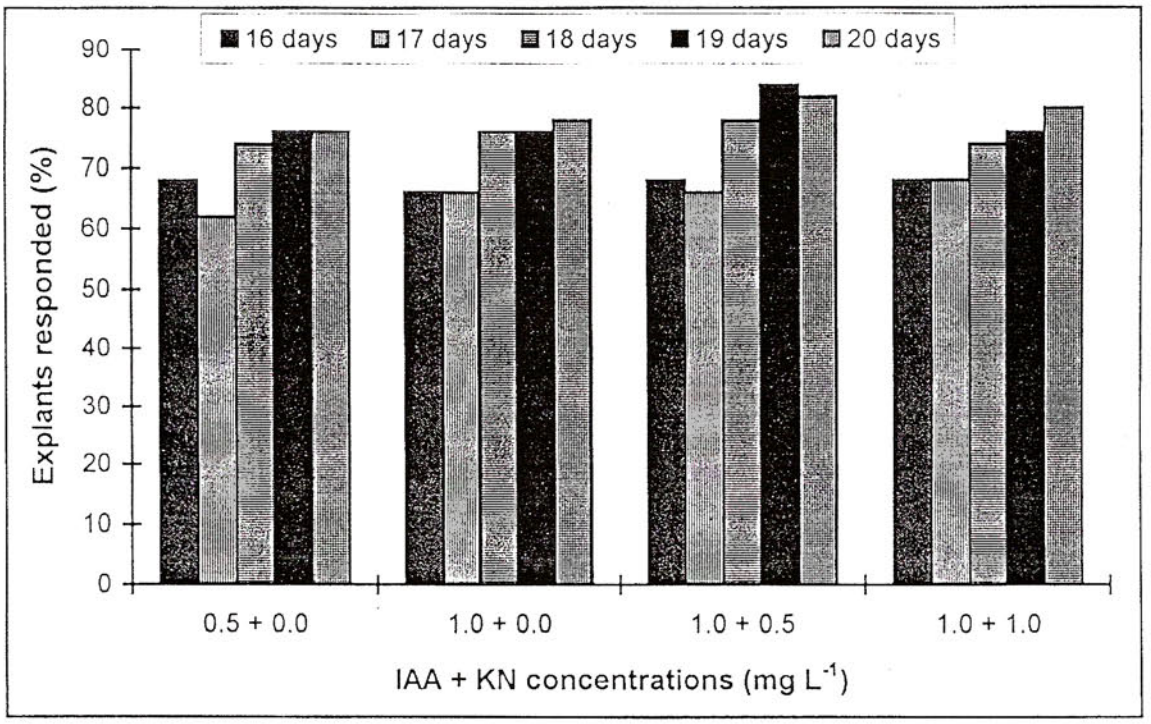
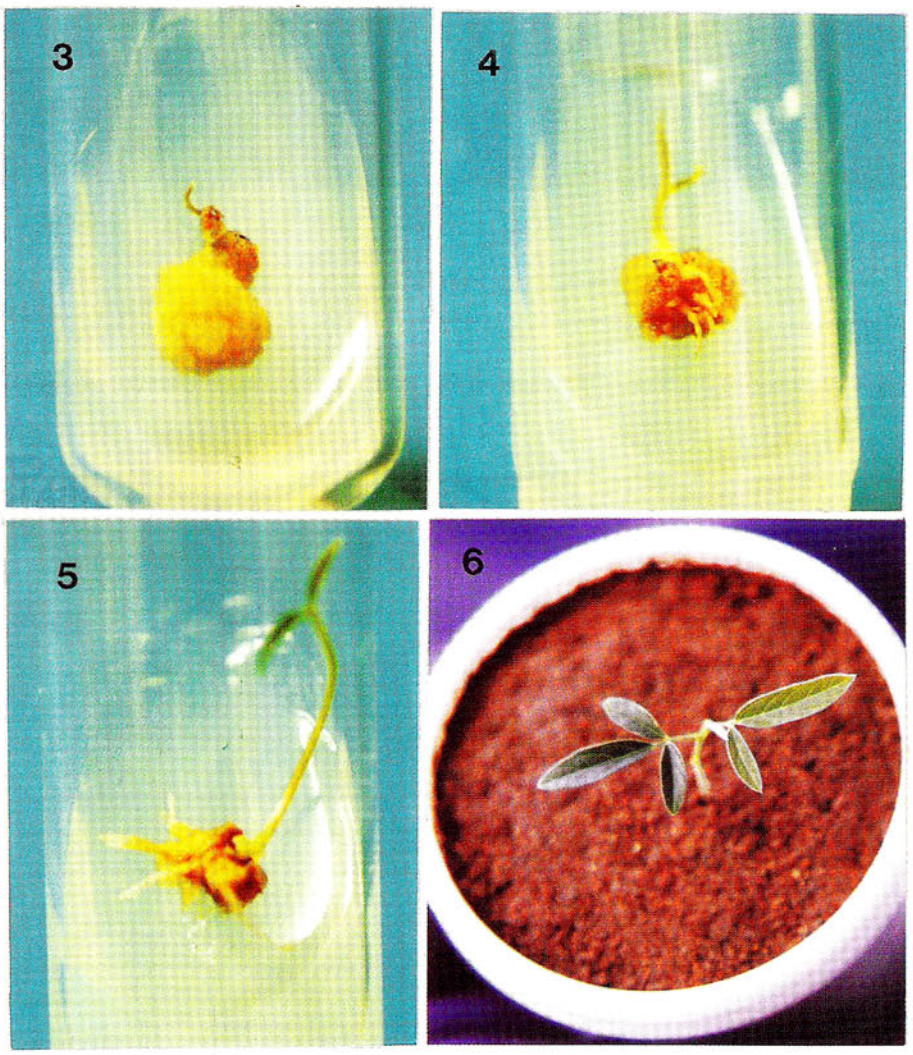


Fig. 2: Morphogenic response of Pigeonpea immature embryos of 16 to 20 days old cultured on B5 medium.

Table 3. Response of immature hybrid embryos between *C. cajan* (♀) and *C. platycarpus* (♂) cultured on MS + IAA (1.0 mgL^{-1}) + KN (0.5 mgL^{-1}).

Age of explant*	Number of embryos cultured	Nature of response				Remarks
		Sw	C	Sh	R	
> 7	25@	+	+	-	-	The explants turned brown within a week.
7-10	22	+	++	-	-	Some of the explants showed swelling and others turned brown.
11-15	19	-	++	+	-	A few embryos produced callus and others showed swelling only.
16-20	15	-	+	++	-	Healthy plantlets are obtained (i.e. plantlets with small shoot and root with scanty callus).

*Days after pollination; Sw = Swelling; C = Callus; Sh = Shoot; R = Root.
'-' = No response; + = Moderate growth; ++ = Better growth; @ = Ovules cultured after cross pollination.



Figs. 3 to 6: Response of immature hybrid embryos of *C. cajan* and *C. platycarpus* cultured on MS medium containing IAA (1.0 mgL^{-1}) + KN (0.5 mg L^{-1}). Fig. 3: Three-week-old culture showing profuse callusing with shoot initiation from 15-day-old explant. Fig. 4: Three-week-old culture from 16-day-old explant showing healthy shoot and small roots. Fig. 5: Three-week-old culture from 19-day-old explant developed into complete plantlet. Fig. 6 : Five-week-old hybrid plant transferred to the hardening mixture of sterilised soil and compost (1:1).

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以組織培養法挽救 *Cajanus cajan* (L) Millspaugh × *Cajanus platycarpus* (Benth.) van der Maesen 之雜種胚

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摘　　要

由於 *Cajanus cajan* 和 *Cajanus platycarpus* 這兩種豆科植物在交配後，其雜種胚胎在發育過程中會發生合子後不親合(post-zygotic)障礙，所以無法利用傳統育種方法將同屬之野生種的基因轉入 Cajanus cajan 而達到改善遺傳性狀的目的。本文利用離體(in vitro)胚培養技術能夠成功地拯救在生體中(in vivo)命定會夭折的雜種胚。取 11 至 15 天大的雜種胚進行培養，不論是在 MS 或 B5 基本培養基中加入不同之 2,4D/IAA 和 kinetin 組合的條件下，均只能得到不分化的癒傷組織；而 16 至 20 天大的胚則可在含 1.0mgL^{-1} IAA + 0.5/1.0mgL^{-1} Kinetin 的 MS 或 B5 培養基上，成功地發育成小苗。由此得知，挽救雜種胚的成功，除了賀爾蒙的濃度與組合有影響外，主要乃取決於外植體的胚齡大小。

關鍵詞：*Cajanus cajan*、*Cajanus platycarpus*、未成熟胚、胚的挽救。

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