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INDUCTION OF MULTIPLE SHOOTS *VIA* ORGANOGENESIS AND PLANT REGENERATION FROM COTYLEDONS OF PIGEONPEA (*CAJANUS CAJAN* L)

CH.SUREKHA and A.ARUNDHATI*
Department of Biochemistry, College of Science, GITAM, Visakhapatnam, India.
*Department of Botany, Andhra University, Visakhapatnam, India.
Email: challa_surekha@yahoo.co.in

Plant regeneration *via* multiple shoot induction from mature cotyledons of pigeonpea (*Cajanus cajan*) has been achieved. The frequency of shoot bud regeneration is influenced by genotype and concentration of phytohormones used. Maximum shoot bud differentiation was obtained on MS medium supplemented with 2mg/l BAP and 0.1mg/l Kn. Elongation of multiple shoots was obtained on MS medium supplemented with 1mg/l BAP, 3 mg/l GA_3 and 0.1mg/l NAA. Shoots longer than 3cm were successfully rooted on MS medium supplemented with 1mg/l NAA. Our observations indicate that regeneration of shoot was obtained through organogenesis. Hence, cotyledons can be successfully used in the production of transgenic pigeonpea plants.

Keywords: Cotyledons; Multiple shoot induction; Pigeonpea.

Introduction

Pigenopea (*Cajanus cajan* L) is an important high protein grain legume widely cultivated in the tropics and subtropics. Genetic improvement of this crop has been achieved to saturation point through conventional plant breeding methods for improvement of different characters. But further improvement is required in the areas of combating with biotic and abiotic stress problems. This can be achieved through non-conventional genetic engineering methods. The introduction of foreign genes and developing a transgenic pigeonpea require suitable, consistent regeneration systems. Although there are several reports on various regeneration systems, a general picture regarding suitability of different explants for transgenic production is not reported. There are reports in *Cajanus cajan*, on regeneration through multiple shoot induction from cotyledonary nodes[1-5], cotyledon[3-4,6-8] and leaf[1,3,9]. Regeneration from shoot tip[2,10] and seedling explants[3,8,11] is also reported in pigeonpea.

The response of mature cotyledons of three varieties of pigeonpea to different phytohormone supplemented media and its suitability for use in transgenic production is reported in the present paper.

Materials and Methods

Seeds of *Cajanus cajan* viz. LRG 30, ICPL 87 and ICPL 85063 were used. Seeds were washed sequentially in dilute soap solution for 5 minutes, running tap water and double distilled water. Seeds surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution were rinsed thoroughly with sterile distilled water and soaked overnight in sterile double distilled water, whose cotyledons were used for testing the morphogenetic potential.

The excised cotyledons were cultured on MS medium (MS), MS supplemented with 2mg/l BAP + 0.1 mg/l Kn ($MSBK_1$); 5mg/l BAP + 0.1mg/l Kn ($MSBK_2$); 10mg/l BAP + 0.1mg/l Kn ($MSBK_3$); 20 mg/l BAP + 0.1mg/l Kn ($MSBK_4$); 2 mg/l BAP (MSB) and 2mg/l 2,4-D (MSD). Cultures were maintained under white fluorescent light (3000lux)at 25±2°C temperature under 16 hr/8hr light/ dark photoperiod.

Cytology - One month old cotyledon segments in culture were fixed in methanol and acetic acid (3:1 ratio) for 24hrs and stored in 70% methanol. Serial sections of the callus developed on MSB and $MSBK_{1-4}$ were stained with Safranin and observed under light microscope.

Buds and shoot clumps from cotyledon explants were subcultured on elongation medium (MS with 1mg/l BAP, 3mg/l GA_3 and 0.1mg/l NAA). The shoots longer than 3cm were cut and transferred to ½ strength MS medium containing 1mg/l NAA for rooting. The well rooted plantlets were acclimatized.

Results and Discussion

The cultured cotyledons swelled up and turned green

Table 1. Comparative response of cotyledon explants of three pigeonpea genotypes for multiple shoot induction on different BAP + Kinetin compositions.

Medium composition	Concentration of BAP (in mg/l)	Concentration of Kinetin (in mg/l)	LRG 30		ICPL 87		ICPL 85063	
			Number responded (in %)	Mean number of shoots ±SE	Number responded (in %)	Mean number of shoots ±SE	Number responded (in%)	Mean number of shoots ±SE
MS	0.0	0.0	70/100 (70.0%)	2.0±1.0	75/100 (75.0%)	2.0±1.0	72/100 (72.0%)	2.0±1.0
$MSBK_1$	2.0	0.1	75/100 (75.0%)	13.5±1.8	82/100 (82.0%)	19.0±1.4	78/100 (78.0%)	17.0±2.5
$MSBK_2$	5.0	0.1	75/100 (75.0%)	13.0±1.1	74/100 (74.0%)	17.5±4.0	74/100 (74.0%)	16.0±2.1
$MSBK_3$	10.0	0.1	56/100 (56.0%)	5.0±0.7	86/100 (86.0%)	7.0±0.8	89/100 (89.0%)	6.0±0.5
$MSBK_4$	20.0	0.1	50/100 (50.0%)	4.5±1.0	50/100 (50.0%)	6.0±0.5	45/100 (45.0%)	5.0±1.2

MS : MS medium.
$MSBK_1$: MS medium supplemented with 2mg/l BAP and 0.1mg/l Kinetin.
$MSBK_2$: MS medium supplemented with 5mg/l BAP and 0.1mg/l Kinetin.
$MSBK_3$: MS medium supplemented with 10mg/l BAP and 0.1mg/l Kinetin.
$MSBK_4$: MS medium supplemented with 20mg/l BAP and 0.1mg/l Kinetin.

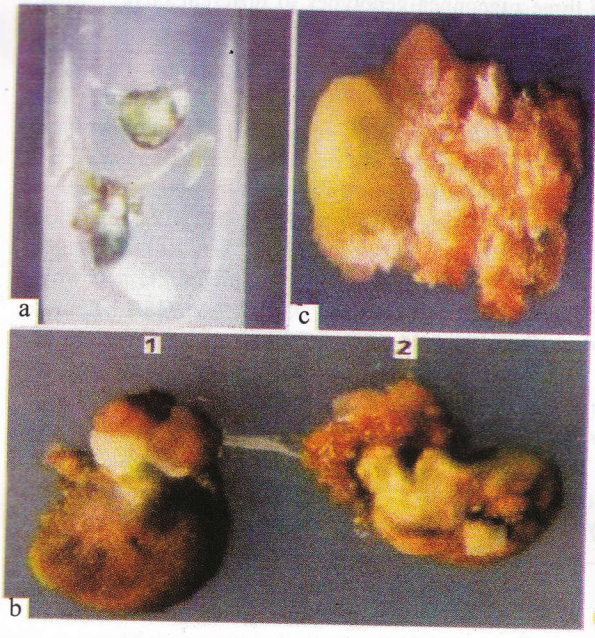

Fig. 1. Excised cotyledons of one month old on different media.
a) Cotyledon on MS medium; b) Cotyledon on MSB (1) and MSBK (2) media; c) Cotyledon on MSD medium.

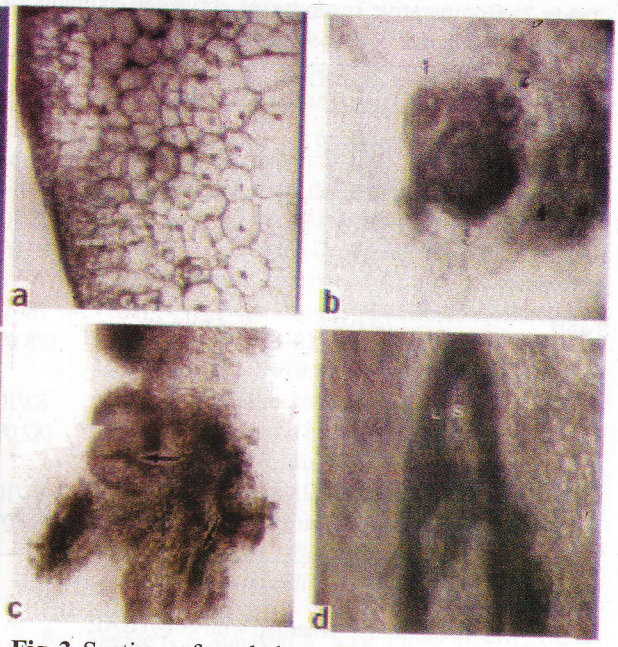

Fig. 3. Sections of cotyledon callus
a) Section of control cotyledon at the time of culture; b) Sections of callus showing shoot tip (S) surrounded by leaf primordia (L); c) Developing shoots from callus each with independent vasculature; d) Callus showing four developing shoot primordia.

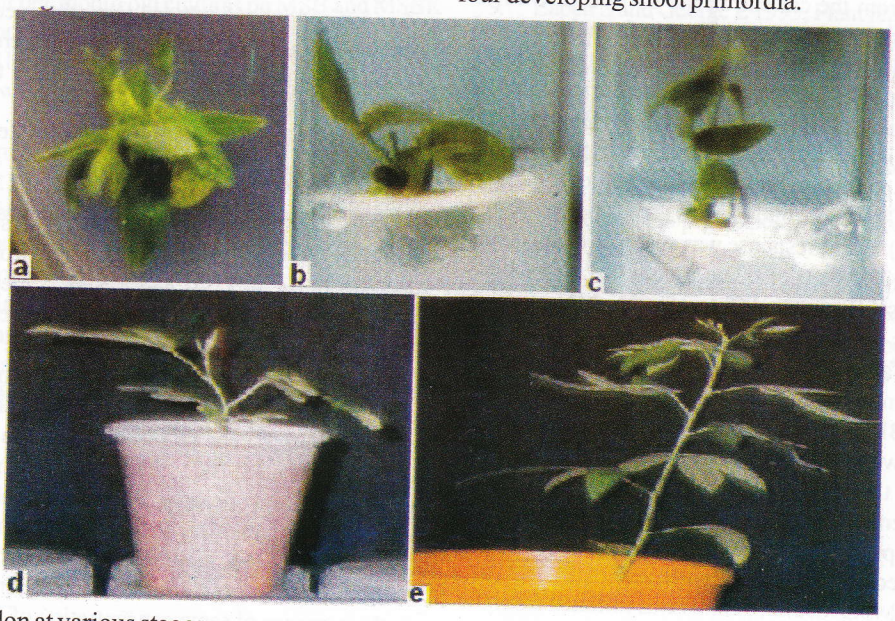

Fig. 2. Cotyledon at various stages
a) Cotyledon cultured on MSBK1 showing multiple shoots after two months; b) Cultured cotyledons on MSB showing multiple shoots; c) Segments of two months old cotyledon with shoots subcultured for elongation; d, e) Elongated shoots kept for rooting (d), Vermiculture (e) Soil.

Table 2. Comparative response of cotyledon explants of three pigeonpea genotypes to different phytohormone compositions.

Medium composition	LRG 30		ICPL 87		ICPL 85063	
	Number responded (in %)	Mean Number of shoots±SE	Number responded (in %)	Mean Number of shoots±SE	Number responded (in %)	Mean number of shoots±SE
MS	75/100 (75.0%)	2.0±0.0	85/100 (85.0%)	2.0±0.0	82/100 (82.0%)	2.0±0.0
MSB_2	72/100 (72.0%)	14.0±2.0	78/100 (78.0%)	18.0±2.0	78/100 (78.0%)	15.0±1.5
MSBK	75/100 (75.0%)	13.5±1.8	82/100 (82.0%)	19.0±1.4	78/100 (78.0%)	17.0±2.5
MSAD	82/100 (82.0%)	0.0	84/100 (84.0%)	0.0	80/100 (89.0%)	0.0

MS: MS basal medium.
MSB_2: MS medium supplemented with 2mg/l BAP.
MSBK: MS medium supplemented with 2mg/l BAP and 0.1mg/l Kinetin.
MSD: MS medium supplemented with 2mg/l 2,4-D.

within a week on all media. Callus initiation was observed on all media from the cut end of the cotyledon. On MS medium, two shoots developed directly from the cut end (Table 1 and 2, Fig.1a), while on MSB (Fig.1b-1) and $MSBK_1$ – $MSBK_4$ (Fig.1b-2) media, shoot buds initiated on the cotyledon surface and callus increased within 3 to 4 weeks. The callus which was in direct contact with the medium turned brown and white buds developed from the callus in 5-6 weeks. The white buds turned green and developed leaves (Fig.2a). Same response was observed on all media (MSB, $MSBK_1$- $MSBK_4$). The number of shoot buds developed was different on different media. More number of shoot buds were produced on $MSBK_1$ than on other media used (Table 1).

The number of shoot buds produced was nearly equal on $MSBK_1$ and MSB media. But the proliferation of shoot buds was fast on MSBK media than on MSB medium. All three varieties showed similar response to various concentrations of hormones used. However, ICPL 87 genotype produced more number of shoots on all the media when compared to other varieties.

On MSD medium, callus was initiated from the cut end of cotyledon, which is white in colour and friable (Fig.1c). The callus increased in 3-4 weeks. No multiple shoot buds were observed (Table 2).

The clumps of shoot buds developed on MSB and $MSBK_{1-4}$ upon subculture in MS+BAP+GA_3 elongated in 2-3 weeks (Fig.2b). Shoots longer than 3cm when excised and subcultured on rooting medium (Fig.2c), developed roots in a week. The plants were hardened in vermiculate (Fig.2d) and then transferred to soil in pots (Fig.2e).

Cytology - 4-7 differentiating shoot meristems (Fig.3a-d) were observed in sections from cultured cotyledons (1 month old).

Cotyledons from overnight soaked seeds cultured on MS medium developed two shoots each, whereas those on MSB and $MSBK_{1-4}$ media developed multiple shoot buds. This is due to the presence of BAP, which induces multiple shoot initials. The multiple shoot initials decreased with increase in BAP concentration as optimum concentration 2mg/l BAP induced more number of shoot initials. The rate of proliferation of shoot buds was higher on $MSBK_{1-4}$ media. This can be due to the presence of low concentrations of kinetin along with BAP, which induced multiple shoots at a faster rate.

The mean number of shoot buds developed from MSB and $MSBK_1$ media are nearly the same. This is due to the presence of same concentration of BAP (2mg/l) in both the media.

All the three genotypes showed similar response to all concentrations of hormones used. This is due to

genotypic differences. However, ICPL 87 genotype produced more number of shoots on all concentrations of hormones used when compared to other genotypes used.

Mohan *et al*[7] obtained an average of 12.5 in GAUT-82-90 and 20.7 in T-15-15 shoot buds on MS medium supplemented with BAP (2.22-22.2μM) and kinetin (0.1-2.3 μM). In the present study, a range of 13.5± 1.8 to 19.0 ± 1.4 shoots from three genotypes on $MSBK_1$ were obtained. Effect of genotype was indicated from these results. Similar results were observed from cotyledons grown on B5 medium supplemented with 10^{-5}M BAP obtained 5-35 shoots[6]. George and Eapen[4] reported only 26.6% of direct shoot bud development (6 shoots per explant) on MS medium supplemented with 1mg/l BAP and 0.1mg/l IAA with explants from the distal end of cotyledons. Geetha *et al*[3] obtained direct multiple shoot bud regeneration from cotyledons excised from 5-8 day of old seedlings.

Kumar *et al*[10] obtained 3-7 shoots on MS medium supplemented with 0.5mg/l 2,4-D and 2 mg/l BAP from whole cotyledons and nodal halves of cotyledons and 5-18 shoots on Blaydes medium supplemented with 2.5mg/l BAP.

All the reports indicate that BAP is more effective than other cytokinins in inducing multiple shoot initials and 2mg/l BAP was found to be optimal concentration for inducing more number of shoot initials.

Sections taken from callus developed from cotyledons of one month old cultures on MSB and MSBK showed shoot meristems. No intermediate embryonic stages could be seen. Therefore, shoot differentiation from cotyledons was through direct organogenesis.

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