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INVITRO REGENERATION VIA MULTIPLE SHOOT INDUCTION FROM IMMATURE EMBRYOS OF PIGEONPEA (*Cajanus cajan*)

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ABSTRACT:
Pigeonpea is an important grain legume, however the genetic transformation of pigeonpea has been very challenging to date. In the present study, we describe a simple protocol for plantlet regeneration via multiple shoot induction from 12-16 day old immature embryos of pigeonpea cultivar ICPL 87 which can be used for efficient transformation. By optimizing the culture medium, we have achieved organogenesis via multiple shoot induction on Murashige & Skoog's medium (MS) medium containing 2, 3 and 4mg/l benzylaminopurine (BAP) from 12-16 day old immature embryos. MS medium fortified with 0.1mg/l NAA and 0.3mg/l GA_3 was optimum for shoot elongation, while half concentration MS medium fortified with 0.5mg/l naphthalene acetic acid (NAA) favoured rooting.
Keywords: Pigeonpea, immature embryos, multiple shoot induction

INTRODUCTION:

Pigeonpea is an important grain legume widely cultivated in the tropics and sub-tropics as a good source of dietary protein, especially in developing countries. Tissue culture technology of pigeonpea has been under investigation, but regeneration of this crop is still limited to date. Regeneration of pigeonpea can be achieved through organogenesis or embryogenesis.

Stable regenerants in pigeonpea have been reported through organogenesis from apical meristem [1], undifferentiated callus [2, 3], differentiated non-meristamatic tissues like leaf [4 – 10] and various seedling explants such as hypocotyls [6], cotyledons [3, 6, 11,12], cotyledonary nodes [6, 7, 13-18], epicotyls [6, 15,17]. Plant regeneration via somatic embryogenesis has been reported [3, 19, 20] and protoplast regeneration up to callus stage has also been reported [21-23] in pigeonpea.

Plant regeneration from embryos was reported by using immature and hybrid embryos [15, 20],

mature embryonal axes [17, 18, 24], decapitated embryonic axis [25] and segments of embryo [26]. We report the response of immature embryos to culture conditions, which can give higher percentages of regenerants for transformation.

MATERIAL AND METHODS:

Cajanus cajan cv. ICPL 87 is used for studying culture response of 11-16 day old immature embryos towards multiple shoot induction and regeneration. The number of immature embryos used varies depending on the germination percentage and availability of embryos at the same stage of development. Numbering of days after pollination (DAP) is taken as the age of immature embryos.

The green immature pods collected from field grown plants were surface sterilized with detergent for five minutes, followed by 70% ethanol for 1 min, 0.1% $HgCl_2$ for 3 min and then rinsed several times with sterile double distilled water. The green immature pods were aseptically dissected to collect embryos and then inoculated on MS medium [27] and MS medium supplemented with different concentrations of BAP (1, 2, 3, 4 and 5mg/l). The cultures were maintained at 25 ± 2^0C under 16/8hr light /dark photoperiod. Polyvinylpyrrolidone (PVP, 100 mg/l) was used in the medium to prevent browning of the tissues.

Explants with multiple shoots were transferred to culture tubes containing 20ml MS medium supplemented with 0.1mg/l NAA and varying concentrations of GA_3 for 4 weeks to encourage shoot elongation. The number of responding explants and shoots per explant were recorded 30 days after transfer to half MS medium. Mean values were quantified from the data of 50 explants. The experiment was conducted three times.

Shoots >3cm were transferred to rooting media containing half strength MS basal medium with 0.5mg/l NAA. The concentration of NAA in rooting media was gradually eliminated over a period of 1 month. Well rooted plantlets were transferred to paper cups containing sterilized mixture of sandy loam soil and vermiculite (1:1) and watered daily with Hoagland's solution [28]. Later, they were transferred to pots with sterile soil and maintained in poly-house under cool temperature for 10days before shifted to open place.

RESULTS:

Explants swelling at the nodal region was the primary response observed within the first five days of culture on all concentrations of BAP added to MS medium and shoot root initiation was observed in explants cultured on MS basal medium alone (Fig. Ia). Embryos younger to 12 days did not survive on all media.

The primary shoot and root exhibited inhibition of growth and induction of shoot buds was observed from the axillary buds of the swollen node of 12-16 day old immature embryos in 10 days on all concentrations of BAP (Fig. Ib). Five to 10 shoot buds were produced per explant in first 20 days and continued to increase until 40 days. The number of shoots was more on media supplemented with 2mg/l and 3mg/l BAP when compared to other concentrations used. There is no much variation in the frequency of shoot bud induction from 12 to 16 day immature embryos. The regeneration capacity of 12 day old immature embryos was low when compared to 16 day old immature embryos (Table I). The number of shoot buds varied from 12 day old to 16 day old immature embryos and the number increased from 12^{th} day to 16^{th} day.

Multiple shoot buds from 12-16 day old immature embryos elongated on MS medium fortified with 0.1mg/l NAA and 0.3mg/l GA_3 (Table II; Fig Ic) and shoots longer than 3cm developed roots when transferred to half strength MS medium supplemented with 0.5mg/l NAA (Fig. Id). Well rooted plants grew well on sterile vermiculate (Fig Ie), polyhouse conditions and flowered in 5-6 months in the normal short day flowering season of *Cajanus cajan.*

DISCUSSION:

Studies on 11-16 day old immature embryos of ICPL 87 revealed that there is increase in percentage of germination with age of the embryo. This result is not uncommon because the embryo has the potential to germinate and differentiate into a plant was after 12 days for ICPL 87 as understood from our studies. But the results of culture of immature embryos of *Cajanus cajan* by different workers appears to be different. Kumar *et al* [15] reported callus development and low level of plant differentiation in 11-14 day old embryos of three varieties and there is direct differentiation into whole plants in 15-19 day old plants with small quantity callus appearance.

Our results are in concurrence with Kusum Kanta & Padmanabhan [24] who cultured full embryo and embryos cut into two, three segments on Nitsch's basal medium supplemented with casein hydrolysate, coconut milk, kinetin and 2,4-D.

The time window for potential to differentiate into whole plant in *Cajanus cajan* appears to be 12^{th} or 14^{th} day in different varieties. Our result appears to be consistent with several varieties studied by Kumar *et al* [15, 19] with respect to hardening and differentiation which proceeds after 14 days of pollination.

The callus induction in Kumar *et al* [15] could be due to use of 2,4 D in both MS and B5 medium and non induction of callus in our studies is because of use of MS or MSB where the conditions are not suitable for callus induction.

More number of multiple shoot buds were observed at node, it can be explained in terms of apical dominance. Franklin *et al* [18] obtained a different result as multiple shoot induction was obtained from apical region when embryonal axes were inoculated on MS medium supplemented with BAP and NAA. Both embryonal axes and nodal explants on MSB1 medium produced callus and shoot, while only shoots were produced on MSB. This is also consistent with well known phenomenon that both cytokinins and auxins are required for callus induction. Higher cytokinin ratio induces shoots and higher auxin ratio induces root differentiation.

For use in transformation, the immature embryos on MSB medium producing multiple shoots appears to be promising to give higher frequencies of transformants as they have more number of meristamatic cells.

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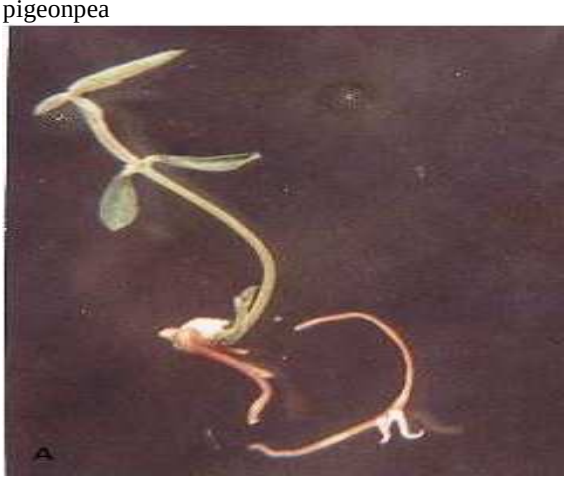
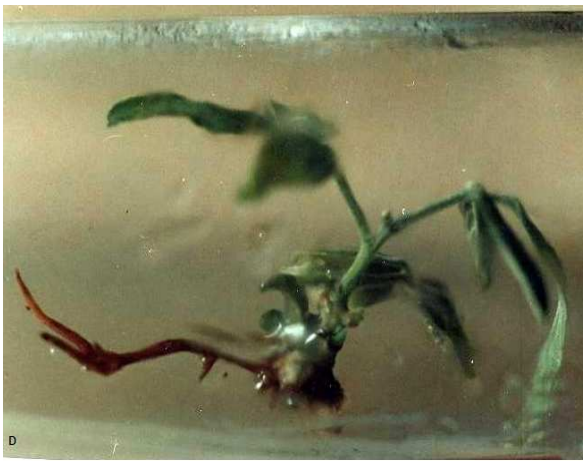
Figures and tables are as follows:

Figure:
Fig 1. *Invitro* regeneration of immature embryos of pigeonpea





A) Immature embryo showing shoot and root after three weeks on MS medium
B) Immature embryo showing multiple shoot buds on MS medium supplemented with 2mg/l BAP
C) Immature embryo on elongation medium
D) Elongated shoot showing root when transferred onto rooting medium
E) Rooted plantlet transferred to sterile vermiculite

Age of Embryos (Days after pollination)	MS+BAP (mg/l)	Explants cultured (No.)	Explants responded		Mean number of shoots /explants
			No.	%	
11	0.0	20	-	-	-
	1.0	20	-	-	-
	2.0	25	-	-	-
	3.0	25	-	-	-
	4.0	25	-	-	-
	5.0	25	-	-	-
12	0.0	20	13	65	2.0±0.12 (8-12)
	1.0	20	13	65	2.1±0.32 (9-12)
	2.0	25	18	72	4.6±0.24 (14-16)
	3.0	25	17	68	4.2±0.10 (12-15)
	4.0	25	14	56	3.2±0.42 (10-13)
	5.0	25	14	56	2.3±0.43 (9-13)
13	0.0	20	14	70	2.0±0.20 (8-12)
	1.0	30	24	80	1.9±0.31 (12-19)
	2.0	40	34	85	4.0±0.21 (11-14)
	3.0	40	32	80	4.1±0.25 (9-11)
	4.0	40	31	77.5	3.8±0.34 (11-12)
	5.0	40	30	75	3.3±0.33 (9-13)
14	0.0	20	15	75	2.5±0.30 (10-12)
	1.0	30	27	90	2.1±0.13 (11-15)
	2.0	50	44	88	5.6±0.32 (13-15)
	3.0	50	43	86	5.2±0.10 (12-15)
	4.0	50	42	84	4.4±0.32 (11-14)
	5.0	50	42	84	3.0±0.43 (11-13)
15	0.0	20	16	80	2.2±0.12 (11-12)
	1.0	30	25	83.3	2.1±0.32 (12-19)
	2.0	50	48	96	5.6±0.21 (14-16)
	3.0	50	45	90	5.2±0.15 (12-15)
	4.0	50	42	84	5.2±0.42 (10-13)
	5.0	50	42	84	3.5±0.4 (10-13)
16	0.0	20	15	75	2.1±0.32 (9-12)
	1.0	30	29	96.6	1.9±0.32 (12-19)
	2.0	50	47	94	6.2±0.22 (15-16)
	3.0	50	47	94	5.8±0.10 (12-15)
	4.0	50	43	86	4.8±0.34 (11-13)
	5.0	50	45	90	4.3±0.45 (12-13)

Table I. Germination percentage and multiple shoot induction of 11-16 day old immature embryos of genotype ICPL 87 on MS media and MS medium supplemented with different concentrations of BAP

Table II Elongation of multiple shoots obtained from 11-16 day old immature embryos cultured on MS medium supplemented with 0.1mg/l NAA and different concentrations of GA_3.

S.No.	MS + 0.1mg/l NAA + GA_3	No. of shoots cultured	Percentage of response	Mean no. of shoots elongated / explants
1	0.0	50	24	2.0±0.12 (9-12)
2	0.1	50	26	2.0±0.11 (7-11)
3	0.2	50	24	4.5±0.22 (8-12)
4	0.3	50	60	6.0±0.32 (15-21)
5	0.4	50	15	1.0±0.5 (9-10)
6	0.5	50	10	1.0±0.1 (7-9)