

High frequency induction of multiple shoots and plant regeneration from seedling explants of pigeonpea (*Cajanus cajan* L.)

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An efficient and direct shoot bud differentiation and multiple shoot induction from seedling explants of pigeonpea (*Cajanus cajan* L.) has been achieved. The frequency of shoot bud regeneration was influenced by the type of explant, genotype and concentrations of cytokinin. Explants, viz. epicotyl, hypocotyl, leaf, cotyledon and cotyledonary nodal segments from 7-day-old seedlings were cultured on MS medium augmented with different concentrations of BAP/kinetin. Among the various concentrations tested, 2.0 mg/l BAP or kinetin was found to be the best for maximum shoot bud differentiation. Percentage as well as the number of shoots per explant showing differentiation of shoot buds was higher on MS media supplemented with BAP compared to kinetin. Elongation of multiple shoots was obtained on MS medium fortified with BAP in combination with NAA and GA₃. The optimal BAP concentration for shoot elongation was 1.0 mg/l. The combination of 1.0 mg/l BAP with 0.1 mg/l NAA increased the number of multiple shoots as well as shoot elongation. Addition of GA₃ along with BAP and NAA combination dramatically enhanced both multiple shoot proliferation and shoot elongation in all the explants. The elongated shoots were successfully rooted on MS medium containing different auxins. Among them IBA at 0.2 mg/l induced maximum frequency of rooting followed by NAA and IAA. Regenerated plants were successfully established in soil where 90–95% of them developed into morphologically normal and fertile plants. This method can thus be advantageously applied in the production of transgenic pigeonpea plants.

LEGUMES are one of the most important groups of crop plants and have been the subject of efforts to improve desirable traits including their *in vitro* culture response. Since legumes are notoriously recalcitrant to regenerate from tissue culture, much effort has been devoted to developing and optimizing efficient *in vitro* regeneration systems to facilitate a variety of technologies¹. The ability to regenerate plants from cultured cells, tissues

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or organs constitutes the basis of producing transgenic crops. Successful regeneration of legume species has been greatly aided by species-specific determination of critical parameters, such as explant source, genotype and media constituents².

Pigeonpea is an important high-protein grain legume of the semi-arid tropics and caters to the protein requirement of a population in the Indian sub-continent. Improvement of pigeonpea cultivars possessing resistance to pests and diseases, tolerance to abiotic stresses and low allergenic proteins in seeds is therefore desirable. The use of transformation techniques for producing new breeding materials that would not be available in the germplasm among cross-compatible species holds much promise in attaining this goal. Genetic improvement of this crop through biotechnological methods has not yet been achieved mainly due to its recalcitrance in culture^{3,4}. Shama Rao and Narayanaswamy⁵ reported

regeneration of pigeonpea from callus cultures of hypocotyls obtained from gamma-irradiated seeds, but failed to observe it in unirradiated controls. The optimal culture conditions for plant regeneration from callus of leaves and cotyledons of pigeonpea were reported by Kumar *et al.*⁶ with an emphasis on creating genetic variability. They also reported that multiple shoots were induced from excised cotyledonary segments when cultured on a medium with BAP. Multiple shoot buds were induced from cotyledonary node explants of pigeonpea⁷. However, in all these cases, the frequency of whole plant regeneration has been low and unsatisfactory. The above mentioned literature suggests that addition of a cytokinin to the regeneration medium could be the keystone for pigeonpea shoot bud differentiation. These workers have not studied the factors that influence plant regeneration in detail. None of the protocols reported in the literature is suitable for the genetic transformation of pigeonpea because of the low frequency of regeneration⁸. The aim of the present investigation was to fill this void. This paper deals with the *in vitro* regeneration and multiple shoot production of *Cajanus cajan* from different seedling explants and the subsequent transfer of the regenerated plantlets to soil.

Materials and methods

Plant material and explant preparation

Seeds of pigeonpea, *Cajanus cajan* L. (cv. Hyderabad C) were obtained from Samrudhi Agroseed Corporation, Bangalore. Healthy and uniform seeds were agitated thoroughly in a dilute Teepol solution for 5 min. Seeds were then rinsed under running tap water. Seeds were then surface-sterilized with 0.1% (W/V) aqueous mercuric chloride solution for 5 min, followed by 4 or 5 rinses of 2 min duration in sterile distilled water. They were germinated aseptically on a medium containing salts and vitamins according to Murashige and Skoog⁹ (MS), 3% (W/V) sucrose and 0.7% (W/V) agar in Magenta jars or on sterile cotton moistened with sterile distilled water in Magenta jars in the dark. Cotyledonary nodes, epicotyls, hypocotyls, leaves and cotyledons were excised from 5 to 8-day-old seedlings and used for tissue culture studies.

Culture medium and conditions

The culture medium was that of MS with 3% (W/V) sucrose. This medium was augmented with 0–5 mg/l of 6-benzylaminopurine (BAP), 0–5 mg/l of kinetin, 0.1–0.5 mg/l α -naphthaleneacetic acid (NAA), 0.1–0.5 mg/l indole-3-acetic acid (IAA), 0.1–0.5 mg/l indole-3-butyric acid (IBA) and 1–5 mg/l gibberellic acid (GA₃) either individually or in combination. The pH of the medium was adjusted to 5.8 prior to adding 8 g/l agar and was autoclaved at 121°C for 15 min. Cultures were maintained at 25 ± 2°C under a 16 h/day photoperiod. White-cool fluorescent tubes were used to obtain 60 μ E m⁻² s⁻¹ light intensity.

Effect of BAP and kinetin for direct shoot bud regeneration

This experiment was designed to compare two cytokinins; BAP and kinetin (0–5 mg/l), with respect to their effects on shoot bud regeneration. Five types of explants such as cotyledonary nodes, epicotyls, hypocotyls, leaves and cotyledons were used. Explants were cultured on MS medium supplemented with different concentrations of BAP and kinetin (0, 1, 2, 3, 4, and 5 mg/l) for shoot bud differentiation in different experiments. After 4 weeks of culture, the number of explants forming shoot buds were counted. Each experiment was replicated thrice, using a total of 50 explants for each treatment.

Optimal BAP concentration for multiple shoot induction and shoot elongation

Buds and shoot clumps were collected following culture initiation, bulked and cultured on MS medium containing different combinations of BAP, NAA and GA₃ for further proliferation and elongation of shoots. Three different treatments, BAP (0.1, 0.5 and 1.0 mg/l) alone, BAP (1.0 mg/l) + NAA (0.01, 0.1, and 0.5 mg/l) and BAP (1.0 mg/l) + NAA (0.1 mg/l) + GA₃ (1, 2, 3, 4 and 5 mg/l) were employed. The cultures were maintained as mentioned previously and subcultured onto the same fresh media after 2 weeks. Shoots longer than 1.0 cm were counted and harvested after 4 weeks and planted on fresh medium. Shoots were again counted after 4 weeks and the data added to the previous data for a final tally of shoot production.

Rooting of elongated shoots and acclimatization

Elongated shoots were excised from all treatments and rooted as detailed below. Shoots collected *in vitro* (> 3 cm long) were transferred to MS medium containing 0.2% (W/V) phytigel (Sigma, USA). The medium was further fortified with 0.1–0.5 mg/l IAA, IBA and NAA individually. Cultures were incubated as described previously. Plantlets with well-developed roots were transferred to magenta jars (7 cm diameter) containing autoclaved soilrite and sand. The potted plants were maintained under the same controlled environmental conditions for 2 weeks. They were watered every 3 days for 15 days. Subsequently they were transferred to earthenware pots (12 cm diameter) containing coarse sand, compost and garden soil and kept in shade for 3 weeks before transferring to the experimental garden.

Statistical analysis

All the experiments were repeated three times and the standard deviation was calculated. Data on shoot bud regeneration, multiple shoot production and rooting were statistically analysed using a completely randomized block design and means were evaluated at the $P = 0.05$ level of significance using Duncan's new multiple range test.

Results and discussion

Surface-sterilized seeds cultured directly on MS basal agar medium without any growth regulators in the jars showed 40–50% germination after 10 days; 95–100% germination was observed after 5 days if the seeds were inoculated on sterile, moistened cotton in the jars. In the present experiment, seed germination percentage was low on agar medium when compared with moistened sterile cotton. This might be due to impurities in the agar and/or the osmotic potential and turgor pressure developed in radicle cells due to high osmolarity^{10,11}. A high percentage of seed germination has been reported on sterile moistened filter paper or cotton in petri dishes

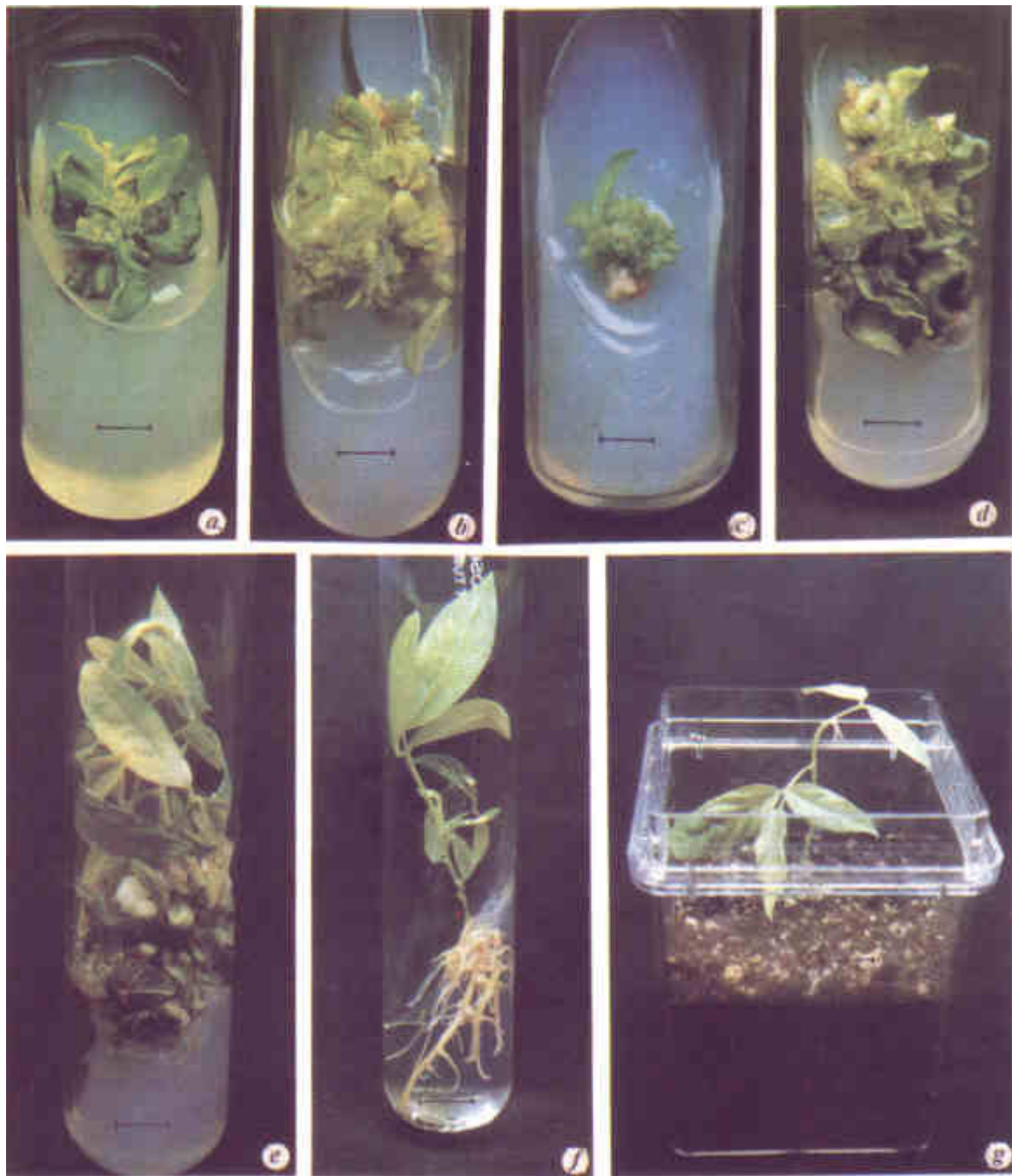


Figure 1. Direct shoot bud differentiation and plant regeneration from different explants of *Cajanus cajan* cv. Hyderabad C. **a**, Shoots developing from leaf explant on MS + 2.0 mg/l BAP (bar = 6.2 mm); **b**, Shoot bud development from hypocotyl explant on MS + 2.0 mg/l BAP (bar = 6.7 mm); **c**, Regeneration of shoot buds from cotyledon explant on MS + 2.0 mg/l kinetin (bar = 5.8 mm); **d**, Shoot bud differentiation from cotyledonary node explant on MS + 2.0 mg/l kinetin (bar = 6.9 mm); **e**, Shoot bud elongation on MS + 1.0 mg/l BAP + 0.1 mg/l NAA + 3.0 mg/l GA₃ (bar = 7.5 mm); **f**, Shoot showing profuse rooting on MS + 0.2 mg/l IBA (bar = 10 mm); **g**, A regenerated plant in Magenta box containing soilrite and sand (bar = 10 mm).

or test tubes in various crop species, including pigeonpea^{6,11-14}. Therefore, seeds were germinated on sterile moistened cotton and used for all the experiments in the present study.

Direct multiple shoot bud induction

The dose of cytokinin is known to be critical in shoot organogenesis¹⁵⁻²⁰. Therefore we compared the response of different seedling explants to various concentrations of BAP and kinetin. Direct shoot bud differentiation was observed after 2 weeks of culture initiation (Figure 1 *a-d*). Multiple shoots were initiated from all the explants after 4 weeks of culture. The frequency of shoot formation was influenced by the type of explant, choice of cytokinin and its dosage. Explants planted on MS without BAP or kinetin did not respond. The highest frequency of shoot bud regeneration was observed from

Table 1. Effect of BAP and kinetin concentration on direct multiple shoot bud regeneration from different seedling explants of pigeonpea after 4 weeks of culture

PGR conc. (mg/l)	Explant response (percentage # mean \pm SD)				
	Cotyledonary node	Epicotyl	Hypocotyl	Cotyledon	Leaf
BAP	00.0 \pm 0.0	00.0 \pm 0.0	00.0 \pm 0.0	00.0 \pm 0.0	00.0 \pm 0.0
1.0	82.5 \pm 2.3 ^b	74.2 \pm 3.2 ^b	68.5 \pm 3.7 ^b	60.3 \pm 2.8 ^b	58.3 \pm 2.4 ^b
2.0	93.2 \pm 4.7 ^a	87.5 \pm 3.8 ^a	80.2 \pm 4.5 ^a	71.4 \pm 3.9 ^a	65.4 \pm 3.1 ^a
3.0	78.3 \pm 3.1 ^c	71.3 \pm 4.2 ^d	65.4 \pm 4.7 ^c	60.3 \pm 3.4 ^b	56.4 \pm 2.8 ^h
4.0	65.4 \pm 2.6 ^c	62.4 \pm 2.3 ^d	59.3 \pm 3.1 ^d	52.5 \pm 1.9 ^c	48.5 \pm 2.0 ^d
5.0	58.7 \pm 2.8 ^f	55.3 \pm 4.1 ^e	50.7 \pm 2.7 ^f	47.3 \pm 1.7 ^d	41.4 \pm 2.2 ^e
Kinetin	00.0 \pm 0.0	00.0 \pm 0.0	00.0 \pm 0.0	00.0 \pm 0.0	00.0 \pm 0.0
1.0	71.2 \pm 3.5 ^d	65.2 \pm 4.2 ^d	61.0 \pm 3.7 ^d	54.1 \pm 2.5 ^c	50.1 \pm 2.9 ^d
2.0	75.4 \pm 4.4 ^k	70.1 \pm 4.8 ^o	64.2 \pm 3.8 ^c	60.2 \pm 3.2 ^h	54.3 \pm 2.1 ^c
3.0	67.3 \pm 3.2 ^e	63.2 \pm 3.7 ^h	59.1 \pm 3.5 ^h	50.4 \pm 2.7 ^f	407.1 \pm 2.2 ^h
4.0	55.1 \pm 2.7 ^f	50.3 \pm 2.9 ^f	56.2 \pm 2.5 ^e	46.1 \pm 3.2 ^e	42.2 \pm 2.7 ^g
5.0	49.2 \pm 2.6 ^g	42.3 \pm 3.1 ^e	38.1 \pm 1.6 ^g	35.3 \pm 2.2 ^f	30.1 \pm 2.0 ^f

Means within a column having the same letter are not statistically significant ($P = 0.05$) according to new Duncan's multiple range test.

Table 2. Effect of different PGRs on development and elongation of multiple shoots of pigeonpea after 8 weeks of culture

Hormone conc. (mg/l)			Explant response (percentage # mean \pm SD)				
			Cotyledonary node	Epicotyl	Hypocotyl	Cotyledon	Leaf
BAP							
0.1			35.3 \pm 2.1 ^l	25.2 \pm 2.4 ^l	21.4 \pm 3.1 ^h	18.5 \pm 1.8 ^h	15.3 \pm 2.8 ^h
0.5			48.4 \pm 3.3 ^h	37.1 \pm 2.6 ^h	35.2 \pm 2.7 ^l	30.2 \pm 3.1 ^g	25.1 \pm 3.8 ^f
1.0			52.3 \pm 2.4 ^h	46.3 \pm 3.2 ^g	41.5 \pm 4.4 ^h	38.9 \pm 2.2 ^c	31.6 \pm 2.6 ^f
2.0			42.3 \pm 3.4 ^h	34.4 \pm 1.9 ^h	30.7 \pm 3.9 ^l	29.2 \pm 1.6 ^g	27.9 \pm 3.1 ^f
BAP NAA							
1.0	0.01		65.7 \pm 4.3 ^e	51.4 \pm 3.7 ^l	47.5 \pm 2.8 ^e	42.3 \pm 2.1 ^c	37.5 \pm 2.3 ^c
1.0	0.1		92.3 \pm 5.4 ^b	64.2 \pm 4.2 ^d	60.7 \pm 3.2 ^d	55.7 \pm 3.8 ^c	45.2 \pm 2.8 ^d
1.0	0.5		72.4 \pm 4.6 ^d	56.9 \pm 3.4 ^e	53.6 \pm 2.9 ^c	47.8 \pm 2.8 ^d	39.3 \pm 2.6 ^c
1.0	1.0		59.4 \pm 3.1 ^f	44.7 \pm 2.8 ^g	40.2 \pm 2.2 ^h	36.2 \pm 3.6 ^f	30.1 \pm 1.9 ^f
BAP NAA GA ₃							
1.0	0.1	1.0	68.4 \pm 3.8 ^o	58.1 \pm 4.5 ^o	51.2 \pm 2.6 ^f	48.1 \pm 3.5 ^d	46.5 \pm 3.9 ^d
1.0	0.1	2.0	83.2 \pm 5.8 ^c	74.5 \pm 4.9 ^h	68.2 \pm 3.5 ^o	63.2 \pm 3.9 ^b	57.5 \pm 2.8 ^h
1.0	0.1	3.0	95.5 \pm 5.7 ^a	98.3 \pm 4.2 ^a	84.2 \pm 4.5 ^a	69.3 \pm 4.2 ^a	62.1 \pm 3.6 ^a
1.0	0.1	4.0	75.3 \pm 5.2 ^d	69.1 \pm 4.7 ^o	64.5 \pm 3.3 ^c	58.5 \pm 3.6 ^c	54.1 \pm 3.2 ^e
1.0	0.1	5.0	66.5 \pm 4.9 ^e	67.2 \pm 5.8 ^c	51.6 \pm 4.3 ^l	46.3 \pm 3.8 ^g	40.4 \pm 2.9 ^g

Means within a column having the same letter are not statistically significant ($P = 0.05$) according to new Duncan's multiple range test.

Table 1. Effect of BAP and kinetin concentration on direct multiple shoot bud regeneration from different seedling explants of pigeonpea after 4 weeks of culture

Table 2. Effect of different PGRs on development and

cotyledonary node explants followed by epicotyl, hypocotyl, cotyledon and leaf explants (Table 1). A maximum of 13–15 shoots were obtained from cotyledonary explant on 2 mg/l BAP. The maximum frequency of shoot formation from cotyledonary node explants was 93.2% on regeneration medium with BAP and 75.4% with kinetin (Table 1). The lowest regeneration frequency from leaf explants induced by BAP was 65.4% and those induced by kinetin 54.3%. Of the two cytokinins tested, BAP was more effective than kinetin in inducing shoot development and multiple shoot induction in all the explants. The regeneration frequency increased with increase in the concentration of cytokinin and 2.0 mg/l was found to be optimal for maximum frequency of shoot bud formation. With cytokinin concentrations above 2.0 mg/l, the frequency of shoot bud regeneration decreased drastically. Franklin *et al.*²¹ obtained a maximum of 49 shoots on 13.31 μ M (3.0 mg/l) BAP with seedling explant which is a combined cotyledonary node and shoot tip and only five shoots with cotyledonary node. BAP is therefore a more effective cytokinin for pigeonpea shoot organogenesis than kinetin. Similar observations have been made for cotyledons of pigeonpea grown on B5 medium⁷. George and Eapen³ reported only 26.6% of direct shoot bud development on MS medium fortified with BAP (1.0 mg/l) and IAA (0.1 mg/l) with explants from the distal end of pigeonpea cotyledons. Eapen and George²² reported low frequency of regeneration from leaf callus cultures of pigeonpea. However, in pigeonpea, Kumar *et al.*⁶ reported 20% of shoot bud regeneration from cotyledonary callus on Blaydes medium with 2.25 mg/l BAP and 14% from leaf callus tissue. Similar results were reported in *Glycine*^{15,18}, *Pisum*¹⁶, *Phaseolus*¹⁷ and chickpea¹⁴. Addition of BAP to MS medium enhanced the frequency of regeneration as well as the number of shoots per cotyledon over the control in mungbean^{19,20}. BAP was the most effective plant growth regulator (PGR) in all these reports, indicating cytokinin specificity for multiple shoot induction.

Multiple shoot proliferation and elongation

In the present study, BAP alone was found to be suitable for both multiple shoot bud induction and proliferation. However, the multiple shoots obtained on various concentrations of BAP failed to elongate on the same medium resulting in rosettes of shoots. Hence it was necessary to develop suitable media for proliferation and elongation of shoot buds. Clumps of multiple shoots were transferred to test tubes containing various PGR combinations for elongation. Multiple shoot proliferation occurred in all five explants on all five concentrations of BAP within 4 weeks (Table 2). The multiple shoot proliferation was found to be highest in the cotyledonary node, followed by epicotyl, hypocotyl, cotyledon and leaf explants. Explants required a higher concentration of BAP (2.0 mg/l) at the initial stage of shoot bud regeneration, but further growth and proliferation of the shoot buds was observed only after subculture to fresh medium with lower level of BAP. Addition of NAA (0.01–1.0 mg/l) enhanced the number of multiple shoots and shoot elongation (Table 2). The optimum level of NAA that promoted the highest number of multiple shoot was 0.01 mg/l. Similar observations were also made for pigeonpea⁴. Shoots elongated and proliferated better than with BAP in mungbean¹⁹. In the present study the problem of shoot elongation was overcome by transferring shoot cultures to a shoot proliferating medium containing NAA and BAP (Figure 1 e). Incorporation of GA₃ (1–5 mg/l) along with BAP (1.0 mg/l) + NAA (0.1 mg/l) markedly increased the number of multiple shoots in all the explants (Table 2). Moreover, shoots showed internodal elongation. The highest number of multiple shoots was scored with BAP (1.0 mg/l) + NAA (0.1 mg/l) + GA₃ (3.0 mg/l). Prakash *et al.*⁴ reported the proliferation of multiple shoot initials from the explant only in the presence of added IAA, suggesting that the cytokinin : auxin ratio

is important for this response. This leads to the conclusion that media with lower concentrations of PGRs favour the elongation of shoot buds. The other two varieties (Vamban and Hindupur) of pigeon pea also responded in the same manner with little variation (data not shown).

Rooting and establishment of plantlets

Elongated and well-developed shoots (> 3 cm long) were excised from the shoot clumps and transferred to MS medium augmented with various concentrations of IAA, NAA and IBA alone for root initiation. Rooting

Table 3. Influence of three auxins on rooting of *in vitro* derived shoots of pigeonpea after 3 weeks of culture

PGR conc. (mg/l)	Explant response (percentage # mean \pm SD)				
	Cotyledonary node	Epicotyl	Hypocotyl	Cotyledon	Leaf
IAA					
0.1	47.2 \pm 2.8 ^d	44.1 \pm 2.2 ^d	37.2 \pm 2.7 ^d	35.3 \pm 1.8 ^d	32.3 \pm 2.2 ^d
0.2	69.2 \pm 4.8 ^a	67.1 \pm 3.8 ^a	68.5 \pm 3.1 ^b	62.5 \pm 2.3 ^b	59.7 \pm 3.1 ^b
0.5	57.3 \pm 4.3 ^d	53.2 \pm 3.2 ^d	49.2 \pm 2.4 ^d	45.2 \pm 2.1 ^d	42.4 \pm 3.8 ^d
NAA					
0.1	50.2 \pm 3.6 ^d	48.7 \pm 4.1 ^d	46.2 \pm 2.1 ^d	41.1 \pm 2.9 ^d	38.4 \pm 2.5 ^d
0.2	77.4 \pm 4.2 ^b	71.3 \pm 5.1 ^b	67.5 \pm 4.3 ^b	60.4 \pm 3.8 ^b	56.4 \pm 3.7 ^b
0.5	60.5 \pm 3.7 ^d	57.3 \pm 3.2 ^d	55.2 \pm 3.0 ^d	48.2 \pm 2.6 ^d	45.2 \pm 3.2 ^d
IBA					
0.1	52.3 \pm 3.4 ^d	50.2 \pm 3.3 ^d	52.1 \pm 2.7 ^d	45.3 \pm 2.8 ^d	45.1 \pm 2.3 ^d
0.2	92.4 \pm 5.1 ^a	85.3 \pm 5.8 ^a	75.3 \pm 4.8 ^a	68.2 \pm 4.2 ^a	63.7 \pm 3.8 ^a
0.5	60.2 \pm 3.6 ^d	63.3 \pm 4.4 ^d	58.6 \pm 2.6 ^d	53.4 \pm 3.2 ^d	50.4 \pm 2.5 ^d

Means within a column having the same letter are not statistically significant ($P = 0.05$) according to new Duncan's multiple range test.

Table 3. Influence of three auxins on rooting of *in vitro* derived shoots of pigeonpea after 3 weeks of culture

the shoots derived from various explants used, those from cotyledonary nodes responded favourably for root initiation; maximum percentage of rooting was observed in all responses to the three auxins (Table 3). The frequency of rooting per shoot was significantly different among the treatments. The percentage of rooting increased with increase in the concentration of auxin. Formation of roots was observed in 69% of shoots on MS + IAA (0.2 mg/l) and 77% rooting of shoots with 0.2 mg/l NAA (Table 3). In pigeonpea, Kumar *et al.*⁶ had observed rooting with IAA (1.0 mg/l) or NAA (0.01 mg/l) and George and Eapen³ had obtained 90% of rooting with 0.2 mg/l NAA. In our work, IBA was found to be best auxin for rooting; 92% of shoots rooted with 0.2 mg/l IBA treatment. Similar results were also reported by Prakash *et al.*⁴ in pigeonpea. Rooting obviously varies in different cultivars.

Upon transfer of the rooted shoots to a hormone free MS liquid medium, the roots showed elongation and put out numerous thin laterals. After hardening, plantlets with fully expanded leaves and well-developed roots (Figure 1 g) were eventually established in the soil. The percent survival of the plantlets in the soil was 90–95%. Almost the same survival percentage was reported by others^{3,4}. The regenerated plants did not show any variation in morphology or growth characteristics when compared with the respective donor plants. The protocol presented here is a substantial improvement over that of the previous findings. In the present investigation, aseptic seedlings were raised on PGR-free media unlike the previous reports in which PGRs (especially BAP) had been used. Regeneration was reported from callus cultures in all the previous reports except by Prakash *et al.*⁴ However, the regeneration frequency was much lower compared to the present study. Prakash *et al.*⁴ have reported an *in vitro* propagation method using cotyledonary node explants, but the rate of multiplication was substantially low.

The results obtained in the present study could be of enormous significance, since data on direct shoot bud induction and multiple shoot production of many Indian cultivars of pigeonpea are not available. This efficient and reliable plant regeneration system via direct shoot

organogenesis can now be exploited for genetic transformation experiments.

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ACKNOWLEDGEMENT. G.L.S. is grateful to CSIR for financial assistance for carrying out this work.

Received 29 July 1998; revised accepted 25 September 1998