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Shoot regeneration from immature cotyledonary nodes in black gram [*Vigna mungo* (L.) Hepper]

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Eighteen-day-old immature cotyledonary node explants (18 d after anthesis) of black gram produced multiple shoots in MS salts+B5 vitamins containing medium in the presence of BA (1.0 mg/L), TDZ (0.1 mg/L) and AdS (15 mg/L). Maximum shoot proliferation (28 shoot/explant) occurred at the end of second subculture after 45 d. Periodic excision of regenerated shoots from explants increased shoot regeneration efficiency during subculture. The combination of TDZ and AdS with BA significantly increased shoot proliferation. Elongation and rooting were performed in GA_3 (0.6 mg/L) and IBA (1.0 mg/L) containing media, respectively. The *in vitro* raised plantlets were acclimatized in green house and successfully transplanted to the field with a survival rate of 60%.

Keywords: axillary meristem, black gram, immature cotyledonary node, subculture, *Vigna mungo*

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Introduction

Vigna mungo (L.) Hepper (black gram) is an important grain legume and one of the main sources of dietary protein for majority of population in developing countries of Asia, Africa and Latin America[1]. Although better agricultural and breeding practices have significantly improved the yield of this pulse over the last decade, productivity has been greatly limited by several viral and bacterial diseases. Since conventional breeding has several constraints[2], *in vitro* culture methods would serve as platform to produce black gram cultivars with desirable characters by genetic engineering techniques.

Legumes in general are recalcitrant to tissue culture and are highly genotype specific[3]. Direct regeneration of black gram from shoottips[4], cotyledonary node[5] and cotyledons[6] has been previously reported from *in vitro* raised seedlings of black gram. However, these reports recorded low shoot regeneration frequency (6-10 shoots/explant). On the other hand, immature cotyledonary node explants produced a high frequency of plant regeneration in several crop species[7]. Therefore, immature cotyledonary nodal explants have been tested for the first time in black gram with an aim to produce higher number of shoots per explant in a reasonably short period.

Materials and Methods

Seeds of *V. mungo* cv.Vamban 3 (a commercial cultivar) were obtained from National Pulses Research Centre, Vamban, Pudukkottai, Tamil Nadu, India. Plants were raised in the experimental field at Bharathidasan University, Tiruchirappalli, Tamilnadu. Eighteen-day-old pods (after anthesis) were washed in running tap water, then soaked in 2% Teepol (3-4 drops per 100 mL dH_2O; a.i. 3.5% sodium hypochlorite, Reckitt and Benckiser, India) for 10 min, washed in dH_2O (5 times) and treated with 0.1% (w/v) $HgCl_2$ (Qualigens, India) for 10 min. Finally. pods were rinsed with sterile dH_2O five times to remove the sterilant. Disinfected pods were cut open aseptically and intact immature seeds were removed, decoated and inoculated in culture tubes (20×150 mm, Borosil, India) containing MS+B5 medium for germination in dark. After 4 d, immature cotyledonary node explants (10-15 mm) were excised and inoculated in 150 mL Erlenmeyer flasks (10 explants/flask) containing MS salts[8] and B5 vitamins[9] supplemented with individual concentrations and combinations of benzyl aminopurine (BA, 0.5-2.0 mg/L) and

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thidiazuron (TDZ, 0.1-0.4 mg /L). adenine sulphate (AdS) at different concentrations (5, 10, 15, 20, 25 mg/L) was used to study its effect on *in vitro* morphogenesis. Different concentrations of auxins (NAA/IAA) individually and in combination with BA were also tested for their shoot induction efficiency. Explants with emerging shoots were subcultured in the same medium (BA+TDZ+AdS) twice at an interval of 15 d each. Shoots that attained 1 cm length were excised from explants and transferred to elongation medium [MS+gibberellic acid (GA_3; 0.2, 0.4, 0.6, 0.8, 1.0 mg/L)]. The elongated shoots (7-8 cm long) were rooted in half strength MS medium with NAA, IAA or IBA (0.5-2.0 mg/L).

Cultures were maintained under white fluorescent light (Philips, India) at a photon flux of 24 µmol m^{-2} s^{-1} for 16/8 h light/dark photoperiod at 25±2ºC. All the media were autoclaved at 120°C for 15 min after adjusting the *p*H to 5.8. Growth regulators were procured from Sigma, St. Louis, USA. TDZ and GA_3 were filter-sterilized (0.2 µm, Pall Gelman Sciences, Mumbai) and added to autoclaved media. For histological studies, explants with emerging shoot buds after one week of culture were fixed in FAA (formalin, acetic acid and ethyl alcohol; 0.5:0.5:9.0, v/v/v) for 48 h and then dehydrated through a graded ethanol xylene series followed by infiltration and embedding in paraffin (58-60ºC). Serial sections of 20-25 µm thickness were cut in a rotary microtome. Sections were affixed to slides, dewaxed, stained with toludene blue, dehydrated and mounted. Photographs were taken with a Nikon E400 microscope with H-III Photographic Unit (Nikon Co., Japan). Every growth regulator treatment was done with 10 explants in ten replicates. A complete randomized design was used in all experiments and analysis of variance and mean separations were carried out using Duncan's Multiple Range Test (DMRT)[10]. Statistical significance was determined at 5% level.

Results and Discussion

In the present study, multiple shoots were induced from axillary meristems of 18-day-old immature cotyledonary node explants (Fig. 1a & b). Shoot induction response varied with the type of growth regulators and their combinations used. In legumes, multiple shoot induction through mature cotyledonary nodes has been widely used for regenerating shoots and for transformation[11]. Axillary meristems of the cotyledonary node explants possess cells that are competent for regeneration and hence are considered as target tissue for gene delivery in legumes. In the present study, 6 shoots/explant were obtained at the end of second subculture in MS medium containing BA (1.0 mg/L) only (Fig. 1c; Table 1). Ignacimuthu *et al*[5] also used BA (13.3 µ*M*) successfully to regenerate multiple shoots from cotyledonary node explant (7shoots/explant). Requirement of BA for induction of multiple shoots has already been reported in other legumes, such as mungbean[12] and chickpea[13]. The factorial combination of BA (1.0 mg/L) with auxins (NAA/IAA) produced basal callusing followed by rhizogenesis and no shoots emerged from the explants (Table 1). Similarly, callusing response was also observed in the presence of NAA (0.1 mg/L) in mungbean[14].

TDZ was tested individually as well as in combination with BA for enhanced multiple shoot induction. Among the different concentrations of TDZ examined, TDZ at 0.1 mg/L produced 8 shoots per explant at the end of second subculture (Table 1). A maximum of 12 shoots per cotyledonary node was observed in MS+B5 medium fortified with a combination of BA (1.0 mg/L) and TDZ (0.1 mg/L) at the end of second subculture (Fig. 1d; Table 1). TDZ has been reported to be the best cytokinin to induce maximum number of shoots in peanut[15] and pigeonpea[16]. Although TDZ at 0.1mg/L induced higher number of multiple shoots, the shoots exhibited stunted growth. To enhance the number of shoots still further with the normal shoot growth, different concentrations of AdS were employed along with BA and TDZ. A combination of AdS (15 mg/L), BA (1.0 mg/L) and TDZ (0.1 mg/L) led to a significant increase in the shoot number (Table 1); At the end of initial culture (15 d after inoculation), 13 shoots per cotyledonary node were obtained, followed by an increase to about 18 shoots per explant at the end of first subculture and about 29 shoots per explant at the end of second subculture (Fig. 1e). The shoots produced in this combination of plant growth regulators showed normal growth. AdS as an additive has generally been used in association with other cytokinins for promoting axillary shoot proliferation and adventitious shoot formation in several plant genera[17]. Periodic excision of shoots from explants as and when they attained a length of 1 cm greatly enhanced the production of multiple shoots in subsequent subcultures from pre-existing meristems of explants. In earlier studies, repeated transfer of explants to fresh medium was found to increase

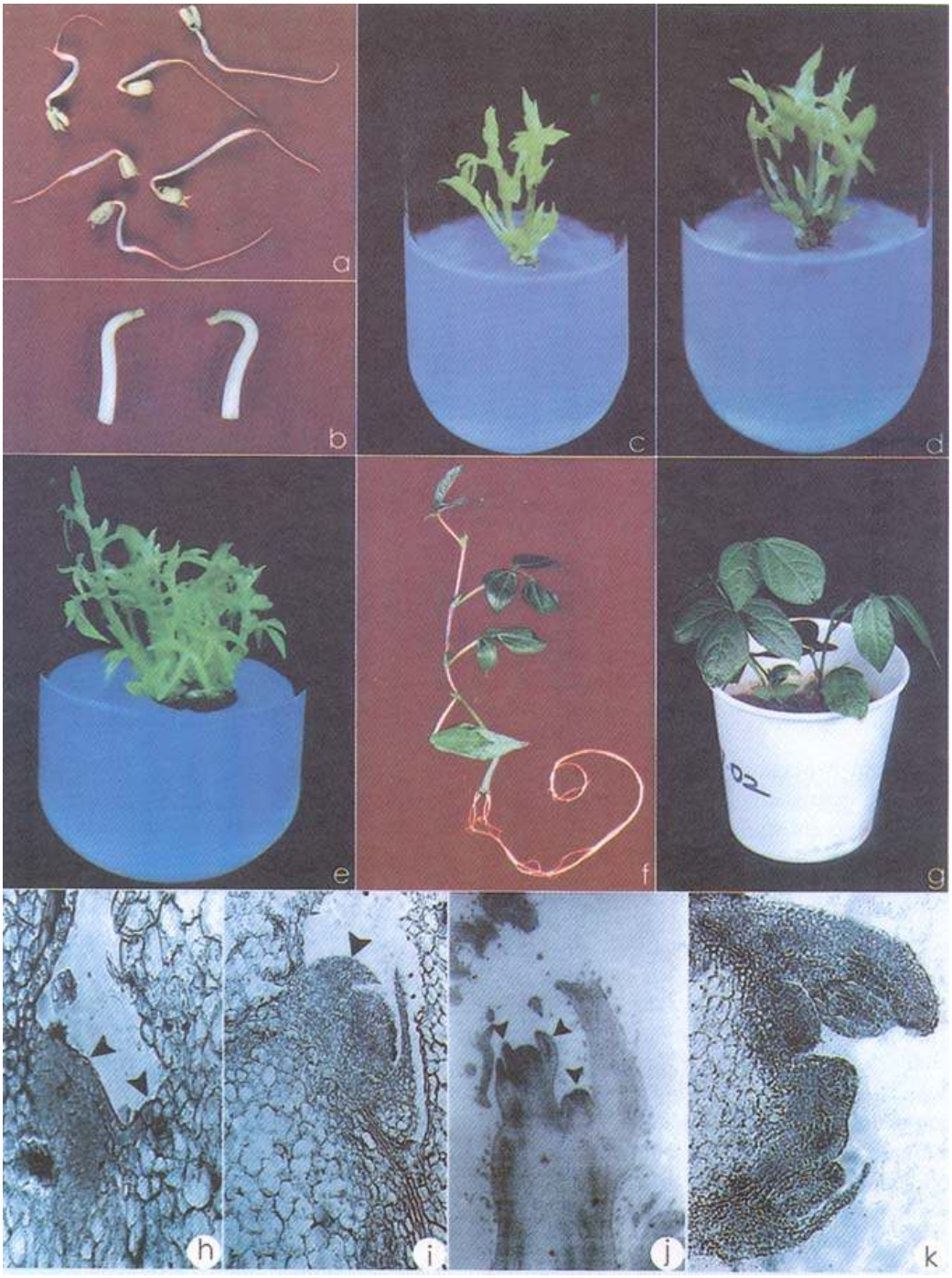

Fig. 1—Shoot regeneration from immature cotyledonary nodes in black gram: a. 18-days-old immature seedlings; b. Immature cotyledonary nodes at the time of inoculation; c. Multiple shoot initiation from immature cotyledonary node explants cultured on MS+B5 medium with BA (1.0 mg/L); d. Multiple shoot formation from immature cotyledonary node explants cultured on MS+B5 medium with BA (1.0 mg/L) + TDZ (0.1 mg/L); e. Multiple shoot formation from immature cotyledonary node explants cultured on MS+B5 medium with BA + (0.1 mg/L) + TDZ (0.1 mg/L) + AdS (15 mg/L); f. Shoot elongation with rooting; g. Plantlet for acclimatization; h. Longitudinal section of the immature cotyledonary node explants at the time of culture showing cell divisions **(►)**; i. Regeneration of shoot buds from the basal regions **(►)**; j. Multiple shoot buds from axillary meristems **(►)**; and k. Multiple shoot buds developed in axillary regions along with nodal regions.

Table 1—Effect of various growth regulators (PGRs) on multiple shoot production from immature cotyledonary node explants of *V. mungo* cv.Vamban 3 on MS + B5 medium

PGR	(mg/L)	Mean no. of shoots/explant in the initial culture (15 days)	Mean no. of shoots/explant during I subculture (15 days)	Mean no. of shoots/explant during II subculture (15 days)
BA	1.0	4 c	5.1 c	6.2 c
TDZ	0.1	2.6 d	3.0 d	8.3 d
AdS	15	0	0	0
BA+TDZ	1.0+0.1	6 b	10 b	12.3 b
BA+AdS	1.0+15	1.5 e	1.5 e	2.6 e
BA+TDZ+AdS	1.0+0.1+15	13 a	17.6 a	28.6 a
BA+IAA	1.0+0.1	0	0	0
BA+NAA	1.0+0.1	0	0	0
BA+IAA+NAA	1.0+0.1+0.1	0	0	0
TDZ+AdS	0.1+15	1 f	1 f	3.5 d f
TDZ+IAA	0.1+0.1	0	0	0
TDZ+NAA	0.1+0.1	0	0	0
TDZ+IAA+NAA	0.1+0.1+0.1	0	0	0
AdS+IAA	15+0.1	0	0	0
AdS+NAA	15+0.1	0	0	0
AdS+IAA+NAA	15+0.1+0.1	0	0	0

For every treatment, PGRs were tested at five different concentrations; BA-0.1, 0.5, 1.0, 2.0, 3.0; TDZ-0.01, 0.05, 0.1, 0.2, 0.3; AdS-5.0, 10.0, 15.0, 20.0, 25.0; IAA-0.05, 0.1, 0.2, 0.3, 0.4; and NAA-0.05, 0.1, 0.2, 0.3, 0.4 mg/L.
Each value represents the treatment means of 10 independent replicates with 10 explants.
Means with the same alphabet in a column are not significantly different according to Duncan's multiple range test at 5% level.

regeneration from explant tissue and activation and conditioning of meristems[18], and this has been supported by the present study.

Subculture was terminated at the end of 45th day as there was no further increase in the number of shoots (data not shown). MS+B5 medium containing BA and TDZ did not favour shoot elongation. Therefore, shoots were subjected to shoot elongation in MS medium containing GA_3 (Fig. 1d). Gibberellic acid at 0.6 mg/L promoted maximum elongation (7.1 cm) of shoots (Table 2). Similar observation was also made in *Vicia faba*[19]. However, shoots did not elongate in MS medium devoid of GA_3 (data not shown).

Longitudinal sections of the immature cotyledonary node explants (at the time of culture) showed divisions which were anticlinal (Fig. 1h). On the fifth day of culture, the cell divisions at the node led to the formation of meristematic regions (Fig. 1i), which after another three days developed into shoot meristems (Fig. 1j). Shoot buds were also formed successfully from actively dividing cells at the base of already regenerated shoots (Fig. 1k).

In vitro raised shoots (7.1 cm long) produced roots in half strength MS medium supplemented individually with IBA/NAA/IAA (Table 3). IBA at 1.0 mg/L produced a maximum number of roots (9/shoot; Fig. 1f), followed by NAA (1.0 mg/L) and IAA (1.5 mg/L; Table 3). IBA played a major role in the root formation of other *Vigna* species, such as *V. radiata*[7,20]. When NAA or IAA was used, callusing occurred at the basal end of shoots together with production of thin delicate roots, thereby reducing the total number of roots (Table 3). Well-rooted plants were transferred to small plastic pots filled with sterilized garden soil and sand (1:1, v/v) for acclimati-

Table 2—Effect of GA_3 on elongation of shoots regenerated from immature cotyledonary node explants of *V. mungo* cv. Vamban 3 on MS medium

GA_3 (mg/l)	Percentage of response	No. of internodes/shoot	Shoot length (cm)
0.2	24 c	2 c	2.9 d
0.4	50 b	4 b	3.9 bc
0.6	67 a	6 a	7.1 a
0.8	24 c	2 c	4.2 b
1.0	14 d	1 d	2.7 df

Each value represents the treatment means of 10 independent replicates with 10 explants.
Means with the same alphabet in a column are not significantly different according to Duncan's multiple range test at 5% level.

Table 3—Effect of auxins on *in vitro* rooting of shoots regenerated from immature cotyledonary explants of *V. mungo* cv Vamban 3 on MS medium

Auxin (mg/l)	% of shoots responded	No. of roots/shoots	Root length (cm)
IBA			
0.5	24 h	3 c	5.7 c
1.0	68 a	7 a	8.9 a
1.5	48 b	4 b	5.2 de
2.0	34 d	3 c	2.5 hi
IAA			
0.5	22 i	2 d	5.5 d
1.0	27 f	3 c	2.6 h
1.5	20 k	2 d	3.0 fg
2.0	21 j	1 f	2.0 j
NAA			
0.5	29 e	3 c	6.5 b
1.0	42 c	4 b	5.2 de
1.5	25 g	3 c	3.5 f
2.0	21 j	2 d	2.5 hi

Each value represents the treatment means of 10 independent replicates with 10 explants.
Means with the same alphabet in a column are not significantly different according to Duncan's multiple range test at 5% level.

zation (Fig. 1f). After 2 weeks, acclimatized plants were transferred to the field and 60% of them survived.

The present study, thus, demonstrates that multiple shoots could be produced from immature cotyledonary node explants with in a short culture period (50-60 d) at a higher frequency than the previous reports in black gram using other explants. Immature cotyledonary nodes (18 d after anthesis) cultured in the presence of BA, TDZ and AdS combination for 2 consecutive subcultures enhanced regeneration frequency significantly. This system is very simple, repeatable and would be more suitable to introduce gene of interest into the genome of black gram via *Agrobacterium*-mediated transformation.

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