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meta-Topolin and β-cyclodextrin enhance multiple shoot and root production in black gram *Vigna mungo* (L.) Hepper

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The recalcitrant nature of black gram is the major constraint of *in vitro* regeneration and *agrobacterium*- mediated genetic transformation, to overcome this, a productive shoot regeneration protocol has been achieved in black gram cultivar T9 using 7-day old cotyledonary node explants excised from *in vitro*- raised black gram seedlings using meta-topolin. An aromatic cytokinin, meta-topolin along with BA (1.5+0.5 mg/L) in 0.50 strength MS medium with 1.5% (w/v) sucrose exhibited a maximum number of multiple shoots (32.0±0.37 shoots/explant) at the end of 6 weeks of culture. The shoots were elongated in (6.40±0.50 cm/shoot) in MS medium supplemented with GA_3 (2.0 mg/L). A maximum number of roots (9.60±0.50/shoot) and root length (11.20±0.73 cm/shoot) were obtained in combination with β-cyclodextrin (a cyclic oligosaccharide; 1.5 mg/L) and IBA (1.5 mg/L). The rooted plantlets were hardened and acclimatized with least mortality rate of 2% in pot mixture consisting red soil:sand:farm yard manure (FYM) (2:1:1) and grown in green house with 85% relative humidity. Ploidy levels were analyzed using flow cytometry which confessed the chromosomal stability in *invitro* raised plants similar to parent plants. This protocol may be useful for producing transgenic black gram with desirable agro-traits in Indian cultivars.

Keywords: Aromatic cytokinin, β-CD, Cotyledonary node, Plant growth regulators, Ploidy

Black gram [*Vigna mungo* (L.) Hepper Fabaceae] is a native cultivated crop in India and now found in many tropical areas of Asia, Africa and Madagascar. India is the world's largest producer and consumer of pulses predominantly tropical and subtropical legumes such as chick pea, black gram, red gram, green gram and lentil[1]. It contains approximately 25-28% protein, 4.5-5.5% ash, 0.5-1.5% oil, 3.5-4.5% fibre and 62-65% carbohydrate on dry weight basis[2]. Black gram is an important source of protein for vegetarian populations. Further, *V. mungo* is an important nitrogen fixing crop used as green manure in agriculture and as nutritive fodder for livestock. For the demand that the black gram has in market, its annual production is low, and not sufficient to meet the recommended dietary allowance. The yield and quality of black gram suffers owing to its sensitivity to a number of biotic (Viral, fungal, bacterial pathogens and insects) and abiotic factors (salinity and drought)[3]. Conventional breeding for crop improvement is not without constraints, and in this context, genetic engineering plays a vital role in crop improvement and crop protection.

Though efforts have been made to standardize *in vitro* regeneration for transformation in *V. mungo*[4], it is highly recalcitrant to regeneration as well as gene[5] transfer method. However, there are many reports on *in vitro* regeneration of black gram using cotyledonary node explants revealing significant variations in the efficiency of multiple shooting and survivability[6]. Therefore, in this study, we tried to evolve a reproducible multiple shoot regeneration protocol that can be used for functional genomics work in black

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gram. Most of the reports available in black gram used cytokinins, such as BA, TDZ, Kn and Zea for multiple shoot regeneration. But regeneration response and multiple shoot number were still lower. Hence, in the present investigation, we tested another type of aromatic cytokinin, meta-topolin in addition to normally used cytokinins. meta-Topolin (mT), originally isolated from Poplar[7] for increased shoot multiplication, maintained histogenic stability, improved rooting efficiency and subsequently reduced production costs in *in vitro* systems[8]. Inclusion of mT in media may ensure efficient commercial *in vitro* propagation of a large number of diverse genotypes[9]. Consequently, mT has been supplemented as a suitable alternative to BA in micropropagation.

β-cyclodextrin (β-CD), a cyclic oligosaccharide compound which is commonly available and economical, appended with auxins/other compounds is reported to improve the rooting efficiency of regenerated shoots [10,11]. The present work reports high frequency plant regeneration in black gram using mT for shoot induction and β-CD for root induction in order to improve its reproducibility.

Material and Methods

The widely grown short duration (60 days) cultivar, T-9 was obtained from National Pulses Research Centre, Vamban, Pudukkottai. Mature uniform healthy seeds were selected and immersed in polysorbate 20 [Polyoxyethylene (20) sorbitan monolaurate] (SRL, India) for 5 min and rinsed thoroughly in running tape water followed by treatment with Bavastin (0.5% w/v) (Carbendazim 50% DF; BASF, New Zealand), a commercial fungicide for 10 min. The seeds were then surface sterilized in 2% sodium hypochlorite (Thermo Fisher Scientific Inc., USA) and 70% alcohol for 3 and 5 min, respectively followed by thorough wash in sterile distilled water for 5 times. They were then imbibed in sterile distilled water for overnight in rotary shaker. The turgid seeds were de-coated aseptically and germinated in Phyta jar (Himedia, India) containing MS solid medium appended with 0.1 mg/L TDZ kept under 16 h light (50 µmol m^{-2} s^{-1}) and 8 h dark at 25±1°C. The above methods followed the earlier reports with some modifications[12].

Effect of cytokinins on shoot induction and multiplication

Cotyledonary nodes (~1.5 cm) were excised from 7-day old *in vitro* grown seedlings and inoculated in culture tubes (2.5×15 mm, Borosil, India) containing MS medium supplemented with various concentrations of cytokinins *viz.* mT (0.5-2.5 mg/L), BA (0.25-1.25 mg/L), KIN (0.5-2.5 mg/L), ZEA (0.5-2.5 mg/L) and TDZ (0.5-2.5 mg/L) in Shoot Induction Medium (SIM). Based on our initial experiment in this study, it was found that mT at 1.5 mg/L exhibited significant improvement in shoot multiplication. Hence, optimum concentration of mT (1.5 mg/L) was combined with different concentrations (0.5-2.5 mg/L) of cytokinins (BA, Kn, Zea and TDZ) to achieve maximum shoot multiplication response. During the initial subculture, the initiated shoot buds were excised carefully using sterile scalpel. All the cultures were maintained at a temperature of 25±2ºCwith fluorescent light with the intensity of 3000 lux for 16/8 h photoperiod[12]. Percentage of multiple shoot induction and mean number of shoots per explant were recorded after 6 weeks of culture. An appropriate control is maintained in all the experiments.

Effect of medium strength and sucrose concentration in shoot multiplication

Different strengths (0.25, 0.50, 0.75, 1.0, 1.25 and 1.50) of MS solid medium were tested to determine their efficiency on shoot multiplication in combination with optimal concentration of mT and BA. Similarly, the impact of carbon source was evaluated by amending different concentrations of sucrose 1.0, 1.5, 2.0, 2.5, 3.0, and 3.50% (w/v) with Cleri gel (0.2%; w/v) (Himedia, India). The percentage of response and number of shoots were recorded after six weeks of culture.

Shoot elongation

The explants along with multiple shoots were excised separately and inoculated on shoot elongation medium (SEM) consisting of MS salts containing various concentration of GA_3 in the range of 1.0-4.0 mg/L. Percentage of response and number of internode/shoot and shoot length were recorded after 4 weeks.

Effect of auxins and β-cyclodextrin on root induction

The elongated shoots, which attained ~5 cm length were selected and transferred to root induction medium (RIM). RIM consists of half strength MS medium fortified with various concentrations (0.5-2.5 mg/L) of IBA, IAA and NAA individually. After four weeks of culture, the optimal concentrations of auxins (IBA 1.5 mg/L, IAA 1.0 mg/L, NAA 1.0 mg/L) were combined individually with various concentrations of β-CD (0.5-2.5 mg/L). Appropriate control was maintained for each experiment and the rooting response such as number of roots/shoot and root length/shoot was recorded after 4 weeks of culture.

Effect of substrates on hardening and acclimatization

Well-rooted shoots were allowed to grow on rooting medium for further 1-2 weeks to maximize rooting efficiency. Then, the *in vitro* regenerated plantlets were removed from the culture vessel, washed gently with double distilled water and transferred to culture tubes containing half strength MS salts for 48 h. The plantlets were finally transferred to paper cups containing autoclaved substrates, Red soil: sand: soilrite (2:1:1), Red soil: sand: vermiculite (2:1:1), Red soil: sand: perlite (2:1:1) and Red soil: sand: farm yard manure (2:1:1), and grown under diffused light (16 h photoperiod). The plantlets were covered with a transparent polythene bags to ensure high humidity and initially irrigated with half strength inorganic salts of MS liquid medium on every alternate days for 3 weeks followed by tap water irrigation. Polyethylene bags were opened gradually. After 4 weeks, acclimatized plants were transferred to pots containing Red soil: sand: farm yard manure (2:1:1) and maintained in green house under 85% relative humidity.

Flow cytometry analysis

Flow cytometry was used to determine the ploidy level of regenerated plants, and their field-grown parent plants. Young leaf samples (20 mg) were suspended in 1.0 mL Tris-$MgCl_2$ solution containing propidium iodide (50 µg/mL) and RNase (50 µg/mL) and chopped with a scalpel to get a fine suspension in 5 cm diameter Petri dishes. The samples were filtered through a nylon mesh (50 µm). Subsequently, the suspension was stored on ice for 10 min prior to analysis, then the amount of nuclear DNA was measured in a flow cytometer (BD FACSAria™, Japan). All the experiments were carried out at least 5 times.

Statistical analysis

The studies were performed in a completely randomized block. The results stated the mean±SE of five repetitions and each treatment consisted of 50 explants. The data were examined using SPSS version 17.0 and substantial variances between means were measured by DMRT at P <0.5.

Results and Discussion

Effect of cytokinins on shoot induction and multiplication

The mean number of multiple shoots per explant, shoot length per explant and their responses varied with type and concentration of cytokinins used (Table 1 and Fig. 1F). In the present investigation, cotyledonary node explant started to bulge after 4-5 days of culture in MS medium fortified with all the types of cytokinins (mT, BA, Kn, Zea, and TDZ). The shoot bud induction was noticed at the axillary region of the explant and these shoot buds were started to produce multiple shoots after 2 weeks of culture. Table 1 explains the effect of cytokinins on shoot multiplication after six weeks. Multiple shoot production responses were considerably enhanced at all the cytokinins tested when compared to control. Of different cytokinins tested, mT

Table 1 — Effect of individual and combination of cytokinins on multiple shoot induction from cotyledonary node explant derived from 7-day old *in vitro* seedlings of black gram cv. T9 on shoot induction medium (SIM) after six weeks of culture

Cytokinins (mg/L)		Percentage of explants responding	Mean number of shoots/responsive explants
Control		20.6±0.50^{t}	2.80±0.37^{l}
mT	0.5	74.40±0.50^{hi}	4.20±0.58^{ijk}
	1.0	82.20±0.37^{e}	5.80±0.37^{gh}
	1.5	90.40±0.67^{b}	9.20±0.37^{d}
	2.0	86.60±0.40^{c}	8.00±0.31^{de}
	2.5	81.60±0.92^{e}	8.20±0.37^{de}
BA	0.25	81.80±0.58^{e}	5.40±0.50^{ghi}
	0.50	84.20±0.58^{d}	7.40±0.50^{ef}
	0.75	82.00±0.70^{e}	6.60±0.50^{fg}
	1.0	79.60±0.50^{f}	4.20±0.37^{ijk}
	1.25	75.20±0.37^{gh}	3.60±0.50^{jkl}
Kn	0.5	67.40±0.40^{j}	4.40± 0.24^{hijk}
	1.0	73.40±0.50^{i}	6.40±0.50^{fg}
	1.5	61.60±0.50^{l}	5.40±0.50^{ghi}
	2.0	54.20±0.58^{n}	4.60±0.40^{hij}
	2.5	49.40±0.24^{o}	4.40±0.50^{hijk}
Zea	0.5	61.40±0.50^{l}	6.00± 0.50^{g}
	1.0	68.80±0.48^{j}	6.60±0.24^{fg}
	1.5	57.40±0.50^{m}	5.20±0.44^{ghi}
	2.0	54.60±0.40^{n}	4.40±0.37^{hijk}
	2.5	47.60±0.40^{p}	4.00±0.24^{ijkl}
TDZ	0.5	63.40±0.50^{k}	5.20±0.37^{ghi}
	1.0	56.40±0.50^{m}	4.60±0.24^{hij}
	1.5	44.80±0.73^{q}	4.20±0.37^{ijk}
	2.0	38.20±0.58^{r}	3.60±0.24^{jkl}
	2.5	29.20±0.37^{s}	3.00±0.31^{kl}
Control		90.40±0.50^{b}	9.20±0.37^{d}
mT + BA (1.5+ 0.5)		93.20±0.37^{a}	18.20±0.58^{a}
mT+Kn (1.5+1.0)		84.60±0.50^{d}	12.40±0.67^{bc}
mT + TDZ (1.5+0.1)		76.20±0.58^{g}	13.40±0.50^{b}
mT + Zea (1.5+1.0)		61.60±0.92^{l}	11.40±0.50^{c}

[Individual cytokinins: Control, Treatment without Plant growth regulators. (For each treatment, 50 explants were used and repeated Five times. Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level); and Combination of cytokinins: Control, SIM containing optimal concentration of mT (1.5 mg/L). (For each treatment, 50 explants were used and repeated five times. Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level)]

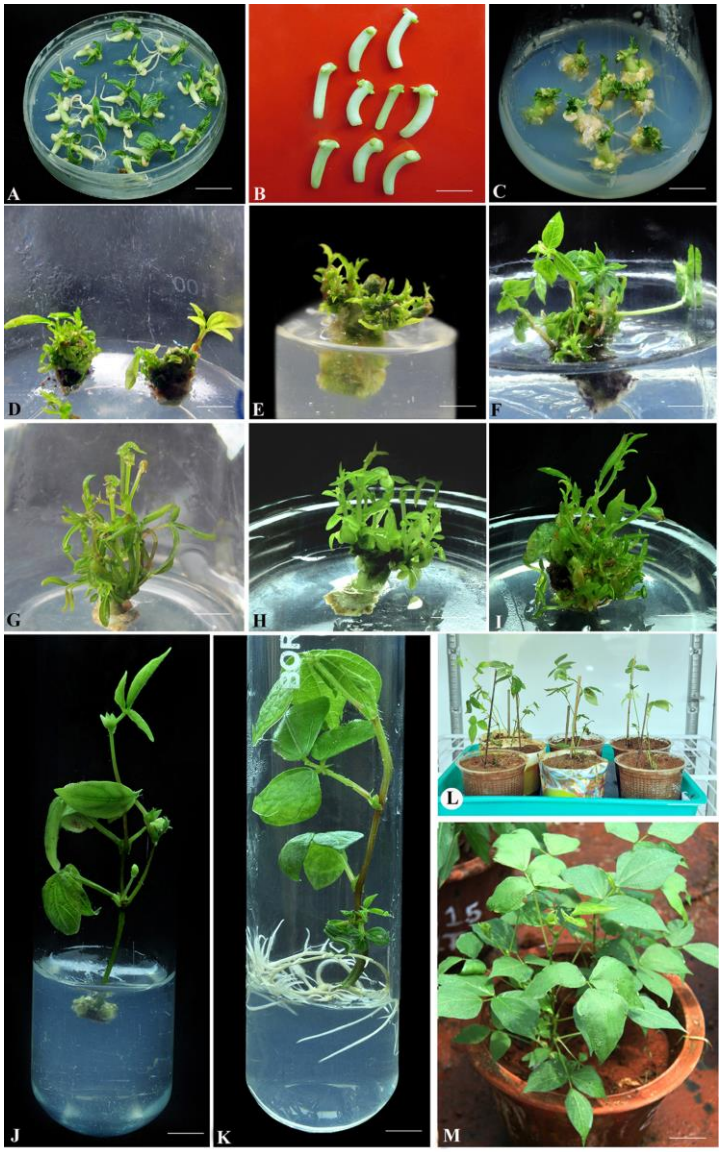

Fig. 1 — High frequency plant regeneration in black gram cv. T9 from cotyledonary node explants. (A) *In vitro*-raised seedlings (7-day-old) (*bar* 0.5 cm); (B) Cotyledonary node explants (*bar* 1cm); (C-E) Multiple shoot induction and proliferation (C. *bar* 0.5 cm, D-E. *bar* 0.5 cm); (F) Production of multiple shoots in MS medium supplemented with mT at 1.5 mg/L) (*bar* 0.1 cm); (G) Multiple shoots regeneration in MS medium appended with mT 1.5 mg/L+0.5 mg/L BA) (*bar* 0.1 cm); (H) Multiple shoots regeneration in 0.5 strength MS medium amended with 1.5 mg/L mT +0.5 mg/L BA) (*bar* 0.1 cm); (I) Multiple shoots production in 0.5 strength MS medium containing 1.5 mg/L mT +0.5 mg/L BA+1.5% sucrose) (*bar* 0.1 cm); (J) Elongated shoots in MS medium supplemented with GA_3 at 2 mg/L (*bar* 0.2 cm); (K) Rooted shoot in MS medium supplemented with 1.5 mg/L IBA+ 1.5 mg/L β-CD (*bar* 0.2 cm); (L) Acclimatized plants in Red soil: Sand: Farm yard manure (2:1:1) (*bar* 0.2 cm); and (M). Hardened plant in green house (*bar* 0.2 cm).

at 1.5 mg/L in SIM was found to be most effective for shoot multiplication and produced a mean number of 9.20±0.37 shoots/explants and the percentage of responding explants was found to be 90.40% (Table 1). In the previous studies, mT was shown to be more effective than BA in inducing shoot proliferation in *Huernia hystrix*[13]. Increase or decrease in the concentrations of mT beyond or below the optimal level (1.5 mg/L) led to the reduction (in numbers) of multiple shoots. There are reports indicating the potential of mT as an effective alternative to BA in micropropagation. meta-Topolin is an hydroxylated analogue of BA with an hydroxyl group attached at its *N*6 side chain which results in the formation of *O*-glucoside metabolites that can be reversibly sequestrated *in planta* to produce active cytokinin

forms when needed[14]. Better multiplication rate and lesser cost, however make mT, the preferred cytokinin in plant micropropagation.

Next to mT, BA (0.5 mg/L) showed better shoot multiplication response (84.20%) and exhibited 7.40±0.50 shoots/explants after 6 weeks of culture. The present result is in agreement with Muruganantham *et.al*.[15] who reported development of 6.4 shoots/explants using immature cotyledonary node of black gram in BA (1.0 mg/L) amended medium. Whereas, Kn (1.0 mg/L) and Zea (1.0 mg/L) produced 6.2 shoots/explants and at 1.0 mg/L 6.6 shoots/explants, respectively. TDZ at 0.5 mg/L evoked low shoot multiplication response i.e., 5 shoots/explant. Results of the present study confirms that mT played an important role in multiple shoot regeneration in *V. mungo* than other cytokinins.

The effect of mT in combination with other best cytokinins is shown in Table 1 in order to depict its role in multiple shoot induction. The present study substantiated the synergistic effect of mT (1.5 mg/L) when combined with other cytokinins. Maximum number of shoots (18.20±0.58 shoots/explant) and their response (93%) were noted in MS medium supplemented with mT at 1.5 mg/L in combination with BA at 0.5 mg/L (Table 1 and Fig. 1G). The micropropagation multiplication process can be increased by combining BA with mT, because the primary metabolite degrades more quickly during acclimatization[16]. Kamínek *et al*.[17] has already compared in standard bioassay, the activity of several cytokinins on ornamental plants and the results proved mT to be more active than BA and the other cytokinins in the class of natural "aromatic cytokinins". Similarly, Souza *et al*.[18] reported mTas an alternative for prevention of oxidative stress in sugarcane micropropagation. In many other treatments, the mean number of shoots and percentage of response were significantly higher when compared to control culture (Table 1). Next to mT (1.5 mg/L) and BA (0.5 mg/L) combination, mT (1.5 mg/L) with TDZ (0.1 mg/L) exhibited a substantial number of shoots (13.40± 0.50/explant) and the percentage of response (76.20%). The response of Kn was on par with TDZ and Zea thereby revealing less response as compared to other cytokinins (Table 1).

Effect of medium strength and sucrose concentration in shoot multiplication

To fine tune media composition to achieve higher multiplication, the optimum mT+BA combination was added to different strengths of MS to find correct

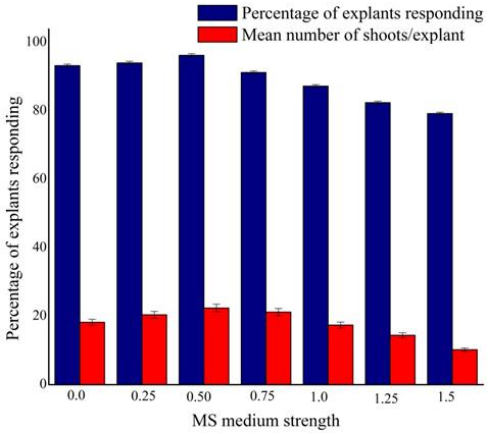

Fig. 2 — Effect of MS medium strength on multiple shoot induction from cotyledonary node explant derived from 7-day-old *in vitro* seedlings of black gram cv. T9 on shoot induction medium (SIM) after 6 weeks of culture. [Control: SIM containing mT 1.5 mg/L and BA 0.5 mg/L. For each treatment, 50 explants were used and repeated five times. Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

medium strength. Among different strengths tested, half-strength MS medium in combination with mT (1.5 mg/L) and BA (0.5 mg/L) showed higher response (96%) (Fig. 2) with mean number of multiple shoots (22±0.31/explant) (Fig. 1H). Full strength MS medium exhibited hyperhydricity due to its high salt concentration, which may triggers oxidative stress. However, the symptoms of hyperhydricity were comparatively reduced when half-strength MS medium was used for multiple shoot induction, which is in accordance with previous report in black gram[19]. This may be due to lower concentration of nitrogen source in the medium. This result was in agreement with Pereira-Pinto *et al*.[20] who noticed that half salt strength of growing medium provided proportionally higher shoot ratio in *Kielmeyera coriacea* regeneration. Further, the half-strength medium in combination with mT and BA was tested with different concentrations of sucrose. Maximum number of shoots (32.0±0.37 shoots/explant) and their response (99%) (Fig. 3) were recorded in half-strength MS medium containing mT (1.5 mg/L) and BA (0.5 mg/L) with 1.5% sucrose after 6 weeks of culture (Fig. 1I). Carbon source plays a vital role in synthesis of cell constituents as a substrate and provides energy for cell growth as well as regulate osmotic pressure[21].

Shoot elongation

The multiple shoots separated from shoot pad were subjected to shoot elongation in SEM. Of different

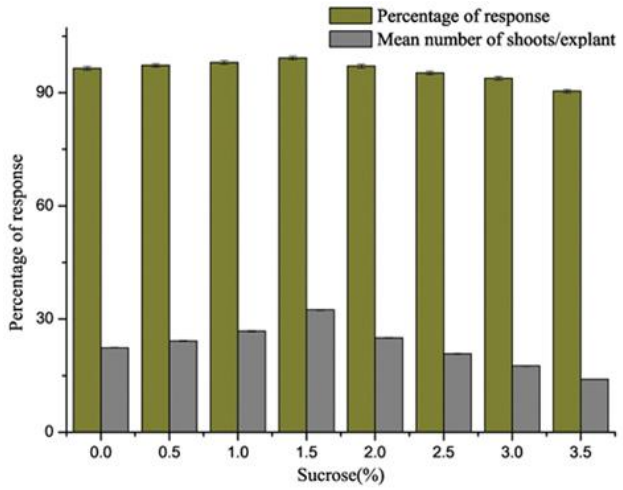

Fig. 3 — Effect of sucrose concentration on multiple shoot induction from cotyledonary node explant derived from 7-day-old *in vitro* seedlings of black gram cv. T9 on shoot induction medium (SIM) after 6 weeks of culture. [Control: SIM containing 0.5 Macro strength + mT 1.5 mg/L and BA 0.5 mg/L. For each treatment, 50 explants were used and repeated five times. Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

concentrations, GA_3 tested at 2.0 mg/L showed higher shoot length (6.40 ± 0.50 cm/shoot), and internode (5.0±0.37/shoot) as well as maximum response (80%) after 30 days of culture (Table 2 and Fig. 1J). Muruganantham *et al*.[15] obtained shoots with six internodes and 67% of response and 7.1 cm shoot length/shoot from immature cotyledon explants at 0.6 mg/L of GA_3. The changes in shoot number as well as length between these reports might be a type of explant used and concentration of GA_3. The shoots elongated in GA_3 were normal and healthy (Fig. 1J). The present report is in agreement with Franklin *et al*.[22] who achieved an efficient shoot elongation in MS medium containing GA_3 only using mature and immature cotyledon explants of soybean.

Effect of auxins and β-cyclodextrin on root induction

Elongated shoots in RIM supplemented with IBA (1.5 mg/L) showed 90% rooting response and produced 6.40±0.50 roots/shoot with a mean root length of 10.40±0.50 cm after four weeks of culture (Table 3). According to previous reports, IBA was found effective for root induction as reported earlier[23] in black gram. Next to IBA, NAA displayed positive response as compared to IAA. In the present study, we combined optimal concentration of each auxin (1.5 mg/L IBA, 1.0 mg/L NAA and IAA each) with different concentration of β-CD in order to increase the root number as well as its response. The result revealed that 1.5 mg/L β-CD along with 1.5 mg/L IBA produce 9.60±0.50 roots/elongated shoot with 11.20±0.73 cm root length (Table 4 and Fig. 1K). This may be due to IBA solubility by forming a complex with β-CDs. The β-CD alone has no effect on rooting as observed by Bettaieb *et al*.[24]. However, they reported that β-CD and IBA combination had good effects on rooting, root length in *Nolina recurvata* Hemsl. The combination proved good for other plant species like *Cynara cardunculus* L. subsp. Scolymus (L) Hegi.[25]

Table 2 — Effect of GA_3 on shoot elongation of regenerated shoots from cotyledonary node explant derived from 7-day-old *in vitro* seedlings of black gram cv. T9 on shoot elongation medium (SEM) after 30 days of culture

GA_3 (mg/L)	Percentage of explants responding	No. of internodes/ shoot	Mean shoot length (cm)
Control	30.60±0.50^{e}	2±0.44^{c}	1.60±0.24^{c}
1.0	60.20±0.37^{d}	3±0.44^{bc}	4.40±0.50^{b}
2.0	80.80±0.86^{a}	5±0.54^{a}	6.40±0.50^{a}
3.0	75.40±0.50^{b}	4±0.44^{ab}	5.60±0.50^{ab}
4.0	64.60±1.0^{c}	2±0.44^{c}	4.40±0.50^{b}

[Control: Treatment without GA_3. For each treatment, 50 shoots were used and repeated five times. Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

Table 3 — Effect of auxins on rooting of elongated shoots of black gram cv. T9 on root induction medium after 4 weeks of culture

Auxins (mg/L)		Rooting response (%)	Mean number of roots/shoot	Mean root length (cm)
Control		30.40±0.74^{l}	2.80±0.37^{fgh}	3.40±0.50^{g}
IBA	0.5	80.60±0.67^{e}	4.80±0.37^{bcd}	8.80±0.37^{b}
	1.0	84.20±0.37^{d}	5.40±0.50^{abc}	8.20±0.37^{bc}
	1.5	90.80±0.58^{b}	6.40±0.50^{a}	10.40±0.50^{a}
	2.0	74.40±0.50^{g}	4.80±0.48^{bcd}	6.40±0.50^{de}
	2.5	63.40±0.50^{i}	2.80±0.37^{fgh}	4.80±0.37^{f}
IAA	0.5	77.20±0.58^{f}	3.80±0.37^{defg}	6.40±0.50^{de}
	1.0	87.40±0.50^{c}	4.40±0.50^{bcde}	7.40±0.50^{cd}
	1.5	70.80±0.58^{h}	4.20±0.58^{bcdef}	6.80±0.58^{d}
	2.0	63.40±0.50^{i}	2.40±0.50^{gh}	5.20±0.37^{ef}
	2.5	53.60±0.40^{j}	1.80±0.37^{h}	2.80±0.37^{g}
NAA	0.5	75.60±0.50^{fg}	4.00±0.44^{cdef}	6.80±0.37^{d}
	1.0	92.60±0.74^{a}	5.60±0.50^{ab}	9.20±0.20^{ab}
	1.5	76.20±0.58^{f}	4.40±0.50^{bcde}	7.40±0.50^{cd}
	2.0	64.80±0.66^{i}	3.20±0.37^{efgh}	5.40±0.50^{ef}
	2.5	43.60±0.50^{k}	2.80±0.37^{fgh}	2.80±0.37^{g}

[Control: Treatment without Plant growth regulator.For each treatment, 50 elongated shoots (>5 cm) were used and repeated five times. Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

Acclimatization

Among the various formulations of substrates tested to acclimatize the *in vitro* rooted plants, Red soil: Sand: Farm yard manure showed better response (Table 5 and Fig. 1L). The percentage of survivability was 98% and the mean shoot length and root length were found to be 45.6±0.40 cm and 12.4±0.40 cm, respectively. This composition of potting mixture was well suited to acclimatize the *in vitro* regenerated black gram. The hardened plantlets were grown normally (Fig. 1M). All the established plants were apparently uniform and did not show any detectable variations. This hardening process has proved to be an efficient alternative to conventional hardening process for acclimatization of *in vitro* raised plants. The survival frequency has been considerably higher after transplantation.

Ploidy analysis

The ploidy level of young leaf segments of the field grown parent plants, and *in vitro* regenerated plants was determined by the flow cytometry analysis. Similar peaks were formed in the two samples (Fig. 4). No significant differences in DNA content were observed in *in vitro* regenerated plants, and field grown parent plants. Flow cytometry is a reliable tool for estimating ploidy level in plants[26]. The protocol, as

Table 4 — Combined effect of auxins and cyclic oligosaccharides (β-CD) on rooting of elongated shoots of black gram cv. T9 on root induction medium (RIM) after 4 weeks of culture

Auxins + Cyclic oligosaccharides (β-CD) (mg/L)	Rooting response (%)	Mean number of roots/shoot	Mean root length (cm)
Control	92.60±0.67^{b}	4.40±0.24^{fghi}	6.40±0.50^{ef}
IBA+β-CD			
1.5+0.5	84.20±0.37^{f}	7.60±0.50^{bc}	8.00±0.44^{cde}
1.5+1.0	93.80±0.37^{b}	8.20±0.37^{b}	9.40±0.50^{bc}
1.5+1.5	98.00±0.44^{a}	9.60±0.50^{a}	11.20±0.73^{a}
1.5+2.0	66.80±0.58^{j}	6.80±0.37^{cd}	7.40±0.67^{de}
1.5+2.5	45.40±0.50^{m}	4.80±0.37^{efgh}	5.40±0.50^{fg}
IAA+β-CD			
1.0+0.5	76.20±0.58^{h}	4.40±0.50^{fghi}	8.20±0.37^{cde}
1.0+1.0	78.00±0.70^{g}	5.00±0.31^{efg}	9.40±0.50^{bc}
1.0+1.5	88.00±0.70^{d}	5.80±0.37^{def}	10.20±0.58^{ab}
1.0+2.0	65.60±0.81^{j}	4.60±0.50^{fgh}	8.20±0.58^{cde}
1.0+2.5	53.40±0.50^{l}	3.00±0.44^{i}	7.60±0.50^{cde}
NAA+β-CD			
1.0+0.5	85.80±0.73^{ef}	5.20±0.58^{efg}	7.40±0.50^{de}
1.0+1.0	90.80±0.73^{c}	6.20±0.58^{de}	8.80±0.37^{bcde}
1.0+1.5	86.60±0.50^{de}	5.40±0.50^{efg}	6.60±0.40^{ef}
1.0+2.0	74.40±0.50^{i}	4.20±0.37^{ghi}	5.20±0.58^{fg}
1.0+2.5	63.40±0.50^{k}	3.40±0.50^{hi}	4.60±0.92^{g}

[Control: Treatment without Plant growth regulator. For each treatment, 50 elongated shoots (>5 cm) were used and repeated five times. Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

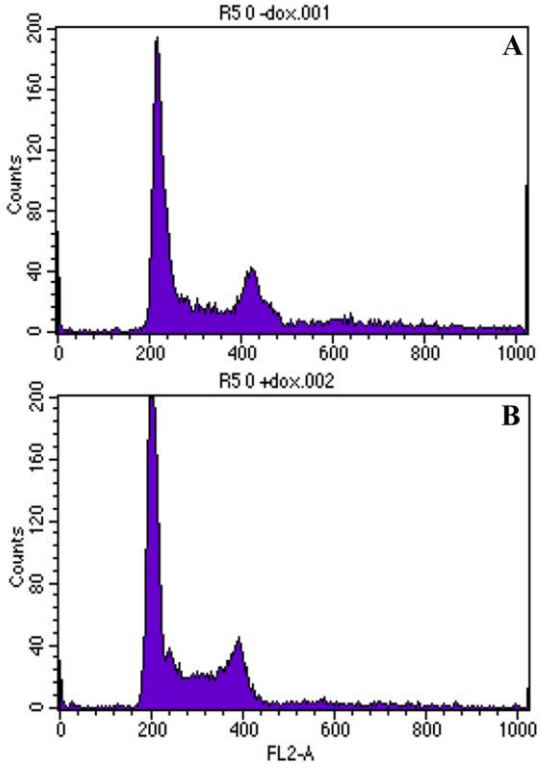

Fig. 4 — Histogram of relative fluorescence intensity of isolated nuclei from black gram cv.T9 obtained from flow cytometric analysis. (A) *Ex vitro* parent plants of black gram cv-T9; and (B) *In vitro* regenerated plants of black gram cv-T9.

Table 5 — Effect of various substrates on acclimatization of *in vitro* raised plants of black gram cv. T9

Substrates	No. of plants transferred	No. of plants successfully acclimatized	Percentage of survivability	Shoot length (cm)	Root length (cm)
Control	50	26^{e}	52 ^{e}	15.2±0.37^{e}	7.2±0.58^{e}
Red soil:sand:soilrite (2:1:1)	50	38^{c}	76^{c}	20.6±0.92^{d}	15.8±0.37^{b}
Red soil:sand:vermiculate (2:1:1)	50	44^{b}	88^{b}	36.2±0.58^{b}	19.2±0.58^{a}
Red soil:sand:Farm yard manure (FYM) (2:1:1)	50	49^{a}	98^{a}	45.6± 0.40^{a}	12.4±0.40^{c}
Red soil:sand:Perlite (2:1:1)	50	33^{d}	66^{d}	29.2±0.37 ^{c}	9.2±0.20 ^{d}

[Data were recorded after 4 weeks. For each treatment, 50 plants were used and repeated five times. Values represent the means±standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

tested in this study, is reliable to produce genetically stable plants for mass multiplication, genetic transformation and germplasm conservation.

Conclusion

The present investigation demonstrated multiple shoot induction from cotyledonary node explants of black gram cv. T-9. The synergistic effect of a cytokinin, mT along with different plant growth regulators (BA, TDZ, Kn and Zea) in combination with a cyclic oligo-saccharide β-cyclodextrin (β-CD) resulted in increased percentage of multiple shoots (32 shoots/explant) and root (9/shoot) production. The present observation is possibly the first report on black gram regeneration system. This protocol can be used to produce large number of regenerated plantlets in a short duration.

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Conflicts of interest

Authors have declared no conflict of interests.

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