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Augmenting *in vitro* Shoot Multiplication by Growth Regulators and Photoperiod in *Vigna mungo* L. (Hepper.)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors GRR and CP designed the experiments. Authors SSS and DS performed the experiments and analyzed the data. Author SSS wrote the draft manuscript. Author GRR finalized the manuscript. All authors have reviewed the manuscript and approved the final manuscript.

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ABSTRACT

An efficient *in vitro* protocol was developed for the mass multiplication of *Vigna mungo* vars. PU30 and PU31, an important legume crop through apical meristems and cotyledonary nodal explants. Both apical meristems and cotyledonary nodal explants were cultured on Murashige and Skoog (MS, 1962) medium fortified with 0.5 – 1.50 mg/L 6- benzyl aminopurine (BA) or Kinetin and 3 % (w/v) sucrose. The rate of multiplication was higher when the cultures were incubated under continuous light (24h) than the 16h photoperiod. The average number of multiple shoots per culture was enhanced from 2.43 to 5.46 in the case of var. PU30 and 3.12 to 5.82 in the case of var.PU31 within 4 weeks of culture under 24h photoperiod. The multiplication rate was enhanced till 5th subculture declined thereafter. Rooting was readily achieved upon transferring the shoots to half-strength MS basal semisolid medium supplemented with 0.1– 1.0 mg/L indole-3-butyric acid (IBA)

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and 2% (w/v) sucrose after 2 weeks of culture. The average number of roots per explants ranged from 3.12 to 5.76. About 80% of regenerated plantlets were hardened in the greenhouse and successfully established in the soil. There is no morphological variation among the regenerated plantlets. This protocol can be used for genetic transformation study for crop improvement of black gram.

Keywords: Black gram; micro propagation; growth regulator.

1. INTRODUCTION

Black gram (*Vigna mungo* L. Hepper) is an important grain legume cultivated mainly in the tropical and subtropical regions of India for its protein-rich edible dry seeds. It is an inexpensive source of nutritionally rich vegetable protein that complements the cereals to provide a balanced diet [1]. Black gram is also one of the main sources of dietary protein for the majority of the population in Asia, Africa, and Latin America. Over the last decade, the yield of black gram has significantly improved but the productivity has been greatly limited by viral, bacterial and fungal diseases like Yellow Mosaic Virus (YMV), Powdery mildew (PM) and Cercospora leaf spost (CLS). Since conventional breeding has several constraints such as eccentric climate, unpredictable weather, and nutrient-poor soil [2,3]. Legumes in general are recalcitrant to *in vitro* culture and are profoundly genotype explicit. Cotyledonary nodes, shoot apices and embryonic axis are the most commonly used explants to regenerate legume crops [4,3]. Plant regeneration either via somatic embryogenesis or direct organogenesis could help in genetic improvement of black gram. Some of the researchers have reported the plant regeneration of black gram from different source of explants such as micro shoot and cotyledonary node [5, 6]. An efficient *in-vitro* regeneration protocol is the primary requirement for the genetic transformation of black gram. Direct *in vitro* organogenesis from explants could be a superior and fast multiplication strategy. The present study optimized an efficient protocol on plant regeneration from the cotyledonary node as well as shoot tip explants of *Vigna mungo* vars. PU30 and PU31 by manipulating growth regulators, photoperiod and culture conditions.

2. MATERIALS AND METHODS

2.1 Source of Explants and Culture Condition

Mature healthy seeds of *Vigna mungo* var. PU30 and PU31 were collected from the Centre for Pulses Research, Berhampur, Odisha University of Agriculture and Technology, Odisha, India. The seeds were rinsed 2 to 3 times with distilled water and treated with 70% (v/v) ethanol for 1 min. Further, the seeds were surface sterilized with 0.1%(w/v) mercuric chloride followed by washing with sterile doubled distilled water for 4 to 5 times to remove any residual disinfectants. These surface-sterilized seeds were aseptically cultured on MS minor and major salts [7] without growth regulator. The cultures were kept in the incubation room at 25±2C under 12h photoperiod (White cool fluorescent tubes with 3000 lux). Five-days-old germinated seedlings were used as a source of explants. Both apical shoots and cotyledonary nodes were excised and aseptically cultured on modified MS medium supplemented with various concentrations and combination of cytokinins (BA (6-benzylaminopurine) or Kn (kinetin); 0, 0.5, 1.0, 1.5 and 2.0 mg/L) and auxins (IAA (indole-3-acetic acid) or NAA (1-napthaleneacetic acid); 0, 0.1, 0.25, 0.5 mg/L) for shoot regeneration. The pH of the media was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl before autoclaving. Routinely, 25 mL of the molten medium was dispensed into culture tubes (25 × 150 mm), plugged with non-absorbent cotton wrapped in one layer of cheesecloth and autoclavedat 121°C and 1.06 kg/cm^2 pressure for 15 min. The cultures were incubated in the culture room with 16h photoperiod at 3000 lux intensity for growth responses. The subculture was made every 4-week interval onto the fresh medium with a similar composition.

2.2 Effect of Photoperiod on Shoot Proliferation

A separate experiment was conducted to achieve the high frequency of plant regeneration from cotyledonary nodal explants of *Vigna mungo* var. PU30 and PU31. The explants were implanted into semi-solid basal MS medium containing 30 g/L sucrose and supplemented with 1.0 mg/L BA and 0.1 mg/L IAA for shoot proliferation and multiplication. The pH of the media was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl before autoclaving. Routinely, 25 mL of the molten

medium was dispensed into culture tubes (25 × 150 mm), plugged with non-absorbent cotton wrapped in one layer of cheesecloth and sterilized at 121°C, and 1.06 kg/cm^2 pressure for 15 min. The cultures were maintained at 25 ± 2°C under 8h, 12h, 16 h photoperiod and 24h (continuous light) from cool, white fluorescent lamps with 3000 lux intensity. The cultures were maintained by regular subcultures at 4-week intervals on fresh medium.

2.3 Induction of Rooting and Acclimatization

For root induction, excised micro-shoots were transferred to the half-strength of basal MS medium supplemented with different concentrations of indole-3-butyric acid (IBA; 0.0, 0.1, 0.25,0.50, 1.25 and 1.50 mg/L) and 2 % (w/v) sucrose. One excised shoot was inoculated in each tube (25 × 150 mm) having 15 ml of the culture media. All the culture tubes were incubated at 25 ± 2 °C under 16h photoperiod with cool, white fluorescent lamps. Rooted *in vitro* propagules were thoroughly washed to remove the adhering the agar gel and transferred to 12 cm plastic cups containing a sterile distilled water for a period of two weeks for acclimatization. Further, the acclimatized plantlets transferred to a mixture of sand, soil, and cow-dung manure in the ratio of 1:1:1 (v/v) and kept in the greenhouse with 85% relative humidity for growth.

2.4 Observation and Statistical Analysis

Fifteen cultures were used per treatment and each experiment was repeated three times. The data represent with the mean of the three independent samples with standard error. The data were analyzed using ANOVA (analysis of variance) followed by DMRT (Duncan's multiple range test) and post-hoc Tukey's HSD (Honest Significant Difference) tests at the significance level of P <0.05.

3. RESULTS

Among two cytokinin tested, BA was the most effective for shoot proliferation and multiplication. The micro shoots were developed from cotyledonary nodal explants and apical meristems on MS medium supplemented with 0.25-1.5 mg/L BA within 2 weeks of culture. There was no sign of shoot proliferation when explants were cultured in media devoid of cytokinin or auxin. The medium having kinetin did not enhance shoot proliferation. Inclusion of IAA or NAA (0.1 to 0.25 mg/L) in BA rich culture medium did not enhance shoot multiplication. Both IAA and NAA at low concentration favour shoot proliferation and multiplication but not at par with cytokinin rich medium. Shoot proliferation and multiplication was achieved in MS medium supplemented with 1.0 mg/l BA (Tables 1 and 2). The maximum number of shoot proliferation and multiplication was observed in cotyledonary node cultured on MS medium supplemented with 1.0 – 1.5 mg/l BA within 4 weeks of culture under 16h photoperiod (Tables 1 and 2). Both apical and cotyledonary meristems were proliferated and elongated to 1.0 –2.5 cm within 4 weeks of culture (Fig. 1A and 1B). At higher concentrations of either BA or Kn or BA+ IAA, the rate of shoot proliferation and multiplication declined. The maximum rate of shoot elongation and proliferation was achieved on MS medium supplemented with 1.0 mg/L BA. Prolonged culture on the proliferation and multiplication media resulted in leaf fall and declined the growth at 16h photoperiod. There were differences among the treatments for both the percentage of cultures with multiple shoots and the mean number of shoots/culture. The highest average percentage of cultures with multiple shoots (86.8 in case of var. PU31 and 82.6 in case of var. PU30) was observed on media containing 1.0 mg/L BA when the cultures were incubated for 4 weeks under 16h photoperiod (Tables 1 and 2).

It was observed that the culture kept in longer photoperiod (24h) favour better growth and raised more number of shoots/explants within 4 weeks of culture (Fig. 2A-C; Fig. 1C). The average number of multiple shoots per culture was enhanced from 2.43 to 5.46 in the case of var.PU30 and 3.12 to 5.82 in the case of var.PU31 within 4 weeks of culture on BA rich medium under 24h photoperiod (Figs. 2B and C). There was no leaf-fall take place from the elongated microshoots, even the culture kept in a prolonged period of up to 6-8 weeks. The rate of multiple shoots were maintained up to 5th subculture on fresh multiple induction medium with similar composition.

Fig. 1A- F. *In vitro* plant regeneration of *Vigna mungo* var. PU 30

A- Cotyledonary nodal explants of V.mungo var.PU30 aseptically cultured on MS medium supplemented with 1.0 mg/L BA after one week of culture; B & C- Multiple shoots developed from cotyledonary nodal explants on MS medium supplemented with 1.0 mg/L BA after 4 weeks of subculture. D - Induction of rooting from in vitro raised shoots cultured on full strength MS medium supplemented with 0.25 mg/L IBA and 2% (w/v) sucrose after 2 weeks of culture. E- Rooted plantlets transferred to sterile distilled water for acclimatization. F- In vitro raised plantlets grown in the soil mixture and bearing flower and fruits after 4 weeks of transfer

Table 1. Effect of MS medium supplemented with different concentrations of growth regulators for shoot multiplication of *Vigna mungo* PU 31 using apical meristems (A) and cotyledonary nodal explants (B) after 4 weeks of subculture on 16h photoperiod at 3000 lux intensity

MS + growth regulators (mg/L)				Percentage of shoot bud proliferation (Mean ± SE)*		No. of shoots/culture (Mean ± SE)*		Av. length (cm) of the shoots/culture (Mean ±SE)*	
BA	**Kn**	**NAA**	**IAA**	**A**	**B**	**A**	**B**	**A**	**B**
0	0	0	0	0	0	0	0	0	0
0.25	0	0	0	20.2 ±1.2 a	26.6 ± 1.2 b	1.24± 0.1 a	2.32±0.2 c	1.51 ±0.2 b	1.62±0.3 b
0.5	0	0	0	48.0±1.6 d, e	62.4± 1.6 h	3.45±0.4 d	4.88±0.5 e	1.72±0.4 c	1.82±0.4 b
1.0	0	0	0	86.8±2.4 j	84.8 ± 2.2 j	4.45±0.3 e	5.26±0.4 f	2.24±0.1 d	2.64±0.3 d
1.5	0	0	0	72.8±1.7 h +	74.6± 1.7 i +	4.34±0.5 e +	4.62±0.5 e+	1.82±0.3 c	2.12±0.2 c
2.0	0	0	0	+	+	+	+	+	+
1.0	0.25	0	0	80.6±2.1 i	78.2 ± 1.4 i	3.25 ±0.2 d	3.84±0.4 d	1.62±0.2 b	1.82±0.2 b
0	0.50	0	0	20.0±1.3 a	22.2 ± 0.9 a	2.24±0.3 c	2.56±0.3 c	1.52±0.3 b	1.72±0.4 b
0	1.0	0	0	42.8±1.1 d+	52.8±1.4 f +	2.14±0.6 c	2.24±0.4 c	1.84±0.4 c	2.01±0.5 c
0	1.5	0	0	54.6±1.7 f+	56.8± 1.2 g +	1.60±0.2 b+	1.41±0.5 b+	1.26± 0.3 a+	1.52±0.1 a+
0	2.0	0	0	+	+	+	+	+	+
1.0	0	0.25	0	66.2± 1.2 g+	24.8± 1.1 h +	1.24±0.4 a+	1.16±0.2 a+	1.42±0.2 a+	1.54±0.3 a+
1.0	0	0.50	0	46.4±0.9 e+	42.6±2.1 d +	1.52±0.3 b+	1.44±0.4 a+	1.22±0.4 a+	1.42±0.4 a+
0	1.0	0.25	0	32.4±1.3 b+	40.2± 1.4 d +	1.66±0.1 b +	1.72±0.3 b+	1.53±0.4 b+	1.65±0.5 b+
0	1.5	0.50	0	30.8± 1.2 b+	36.2± 1.2 c+	1.32±0.2 a+	1.54±0.4 b+	1.45±0.3 a+	1.24±0.6 a+
0.5	0	0	0.25	21.3±1.1 a	27.6±1.0 b	1.12±0.3 a	1.12±0.2 a	1.23±0.2 a	1.45±0.7a
1.0	0	0	0.50	55.6±1.3 f+	48.2±1.4 e+	2.56±0.6 c	1.34±0.5 a+	1.36±0.3 a+	1.56±0.5 a+
0	0.5	0	0.25	18.8±0.9 a	20.8±1.2 a	1.10±0.5 a	1.04±0.2 a	1.42±0.2 a	1.48±0.4 a
0	1.0	0	0.50	36.7±1.1 c+	42.8± 1.1 d +	1.84±0.4 b	1.24±0.3 a+	1.44±0.3 a+	1.54±0.5 a

*(*15 replicates/ treatment; repeated thrice, + - indicate callusing formation)*

Mean value having same letter in a column were not significantly different (P ≤ 0.05) based on Duncan's Multiple Range Test

Table 2. Effect of MS medium supplemented with different concentrations of growth regulators for shoot proliferation of *Vigna mungo* PU30 using apical meristems (A) and cotyledonary nodal explants (B) after 4 weeks of subculture on 16h photoperiod at 3000 lux intensity

MS + growth regulators (mg/L)				Percentage of Shoot bud proliferation (Mean ± SE)*		No. Of shoots/culture (Mean ± SE)*		Av. length (cm) of the shoots/culture (Mean ±SE)*	
BA	**Kn**	**NAA**	**IAA**	**A**	**B**	**A**	**B**	**A**	**B**
0	0	0	0	0	0	0	0	0	0
0.25	0	0	0	18.5 ± 0.9 a	22.4 ± 0.8 a	1.21 ± 0.4 a	1.72 ± 0.5 b	1.21± 0.2 a	1.31 ±0.3 a
0.5	0	0	0	42.8 ± 1.3 c	58.8 ±1.2 d	2.34 ± 0.3 b	4.14 ± 0.4 d	1.51 ±0.4 c	1.68 ±0.2 b
1.0	0	0	0	80.6 ± 1.2 f	82.6 ±1.4 g	4.26 ± 0.7 d	4.86 ± 0.6 e	2.32 ±0.8 e	2.68 ±0.5 d
1.5	0	0	0	74.2 ± 1.3 e+	70.2 ±1.0 e+	4.12 ± 0.7 d+	4.72 ± 0.8 e+	1.81 ±0.6 d	2.14 ± 0.6 c
2.0	0	0	0	+	+	+	+	+	+
1.0	0.25	0	0	72.4 ± 1.4 e	78.4 ±1.2 f	3.00 ± 0.6 c	3.72 ± 0.6 d	1.92 ±0.8 d	2.00 ±0.6 c
0	0.50	0	0	26.4 ± 0.8 b	24.8 ±1.1 a	2.14 ± 0.5 b	2.66 ± 0.5 c	1.50 ±0.6 c	1.62 ±0.7 b
0	1.0	0	0	38.2 ± 1.1 c+	47.7 ±1.3 c+	2.58 ±0.4 c+	2.54 ± 0.6 + c	1.64 ± 0.2 c	1.81 ± 0.5 c
0	1.5	0	0	48.8 ± 1.2 c+	58.2 ± 1.1 d+	2.54 ± 0.5 c+	2.16 ± 0.4 c+	1.22 ± 0.4 a	1.54 ± 0.5 b
0	2.0	0	0	+	+	+	+	+	+
1.0	0	0.25	0	60.8 ± 1.2 d	68.4 ±1.2 e+	1.62 ± 0.3 a+	1.84 ± 0.2 b+	1.42 ± 0.4 b	1.50 ± 0.6b
1.0	0	0.50	0	42. 6± 1.3 c+	48.2 ±1.4 c+	1.58 ± 0.4 a+	1.62 ± 0.6 b+	1.26 ± 0.6 a	1.38 ± 0.3 a
0	1.0	0.25	0	34.2 ± 0.8 b+	42.4 ±1.0 c+	1.68 ± 0.6 b+	1.62 ± 0.4 b+	1.55 ± 0.7 c	1.62 ± 0.4 b
0	1.5	0.50	0	36.4 ± 1.2 b+	40.6 ± 0.9 c+	1.66 ± 0.7 b+	1.60 ± 0.6 b+	1.56 ± 0.6 c	1.45 ± 0.7 a
0.5	0	0	0.25	38.4 ± 1.0 b	36.8± 1.0 b	1.28 ± 0.5 a	1.16 ± 0.4 a	1.12 ± 0.3 a	1.14 ± 0.6 a
1.0	0	0	0.50	62.8 ± 1.4 d+	68.4± 1.6 e+	2.24 ± 0.6 c+	2.34 ± 0.6 c+	2.04 ± 0.5 e	2.15 ± 0.4 c
0	0.5	0	0.25	22.8 ± 0.8 a	28.2± 1.3 a	1.54 ± 0.4 a	1.66 ± 0.4 b	1.14 ± 0.2 a	1.24 ± 0.7 a
0	1.0	0	0.50	42.6 ± 1.0 c+	45.6 ±1.1 c+	1.76 ± 0.6 b+	1.68 ± 0.6 b+	1.36 ± 0.5 b	1.44 ± 0.4 a
1.0	0	0	0.25	54.2 ± 0.9 d+	62.8± 1.4 d+	2.56 ± 0.4 c+	2.68 ± 0.8 c+	1.80 ± 0.7 d	1.92 ± 0.6 c

*(*15 replicates/ treatment; repeated thrice; + - callusing formation)*

Mean value having same letter in a column were not significantly different (P ≤ 0.05) based on Duncan's Multiple Range Test

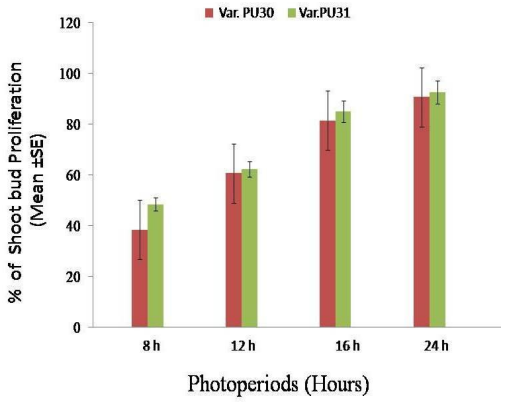
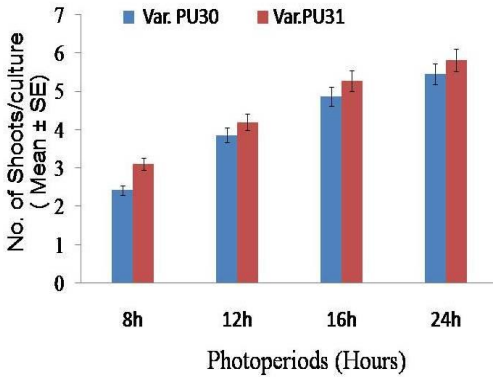


Figure 2A



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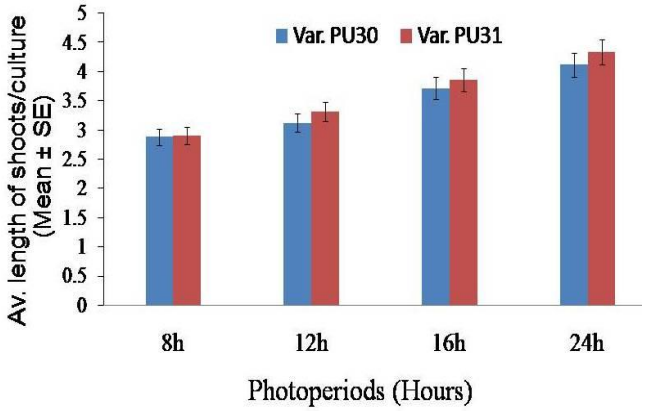

Figure 2C

Fig. 2A-C. Effect of photoperiod regime on shoot proliferation and multiplication of *Vigna mungo* var. PU30 and PU31 cultured on MS medium supplemented with 1.0 mg/L BA + 3% (w/v) sucrose after 4 weeks of subculture. (*15 replicates/ treatment; repeated thrice)

Table.3. Effect of different concentrations of IAA and IBA on root induction from microshoots of *Vigna mungo* var.PU30 and PU31 after 2 weeks of culture

½ MS + IBA (mg/L)	Average percentage of rooting (Mean ± SE)*		Average number of roots/explants (Mean ± SE)*	
	Var. PU30	Var. PU31	Var. PU 30	Var. PU31
0	0	0	0	0
0.1	40.6 ± 1.4 a	46.2 ± 1.2 b	3.23 ± 0.5 a	3.45 ± 0.7 a
0.25	82.8 ± 2.3 d	80.6 ± 2.6 d	4.56 ± 0.9 b	4.78 ± 0.8 c
0.50	66.4 ± 1.3 c	70.4 ± 1.2 c	5.42 ± 0.7 c	5.76 ± 0.5 d
1.00	42.8 ± 1.2 b+	36.8 ± 0.8 a+	3.12 ± 0.6 a +	3.44 ± 0.6 a+
1.25	+	+	+	+
1.50	+	+	+	+

*(*15 replicates/ treatment; repeated thrice, + callusing at the basal end); Mean value having same letter in a column were not significantly different (P ≤ 0.05) based on Duncan's Multiple Range Test*

3.1 Induction of Rooting from Microshoots

The elongated *in vitro* grown shoots were excised and transferred to both full strength MS basal medium supplemented with different concentrations of IBA with reduction of sucrose concentration from 3 to 2% (w/v). It was found that the elongated shoots were rooted on full strength MS basal medium supplemented with various concentrations of IBA (Table 3). There was no positive response when the micro

shoots were cultured without growth regulators. Initiation of roots took place in MS basal medium supplemented with 0.1 to 1.0 mg/L IBA with 2% sucrose within 10days of culture. The shoot growth was also increased within the incubation period. The optimal rooting and growth of micro shoots were observed on medium containing 0.25 mg/L IBA (Fig. 1D). At higher concentrations of IBA, the percentage of rooting was decreased with the increase of basal callusing of the micro shoot. The results also indicate that not only the reduction of rooting ability but also leads to root necrosis with the increase in the concentration of IBA. The percentage of shoots forming roots and days to rooting were varied with different concentrations of IBA (Table 3).

3.2 Plant Acclimatization

The survivability of *in vitro* raised plantlets in the natural environment was a great concern in the optimization of the protocol on shoot multi-plication of *Vigna mungo*, an important pulse crop. About 80% of the rooted plantlets were established in the greenhouse within 4 weeks of culture. The plants were grown well and attained 6 to 8 cm height within 8 weeks of transfer and bearing flower and fruiting (Fig. 1E and F). The acclimatized plants exhibited normal development and no morphological variation was obtained. The step of plant regeneration of *Vigna mungo* is presented in Table 4.

4. DISCUSSION

The present study indicated that it was possible to optimize the regeneration potential of *Vigna mungo* vars. PU30 and PU31 by application of growth regulators and culture conditions. Among the two cytokinins, BA helps for induction and multiplication of shoots derived from apical and cotyledonary nodal explants. The regulatory action of cytokinin and apical dominance helped the *in vitro* shoot induction and multiplication [8]. The maximum shoot proliferation and multiplication were observed both in apical meristems and cotyledonary nodal explants cultured on MS medium supplemented with 1.0 mg/L BA within 4 weeks of culture. These results are supported by the observations from earlier studied in *Vigna* species [9]. At higher concentrations of BA, the rate of shoot proliferation declined. A similar observation was reported by different researchers in various leguminous crops like *Vigna* species [1,10]; *Lens culinaris* [11]; and *Clitoria ternatea* [12]. Mony et al [13] reported that cotyledonary nodal explants of *Vigna mungo* cultured on MS medium containing 1.0 mg/L BA for 10 days followed by transfer to MS medium without BA showed a higher rate of shoot multiplication. They also noted that the least number of shoot proliferation was observed when the explants cultured for 15 days on MS medium containing 10.0 mg/l BA followed by transfer to hormone free MS medium. However, the present study noted that the supplementation of either IAA or NAA in the BA rich medium induces shoot proliferation and multiplication but lower than the BA alone. The interaction of photoperiod and plant growth regulators has a major effect on shoot multiplication as reported by Hashemabadi and Kaviani [14] and Sahoo et al [15]. The findings also indicate that there were significant differences among the four photoperiods (8h, 12h,16h and 24h) for both the percentage of culture developing multiple shoots and the mean numbers of shoots per culture. This might be due to the balancing of the light source, endogenous and exogenous growth regulators, and the ionic concentration of nutrient salts. The cultures kept in continuous light (24h photoperiods) helps higher growth and inhibition of leaf-fall and shoot necrosis. The effect of light on *in vitro* morphogenesis has been reported [16]. Interaction between illumination conditions and growth regulators in shoot differentiation *in vitro* was noted in *Prunus* species [17] and *Vigna* species [15]. It is well known how light affects plant development, especially in *vitro* culture conditions when plants are exposed to continuous light, a condition that can produce significant growth [18-22]. In the present study, it is noted that the multiplication rate was high and stable up to the 7^{th} subculture and declined in subsequent subcultures. This might be due to a balance interaction between the endogenous and the exogenous growth regulators and the ionic concentrations of nutrient salts as reported earlier in other plants [3, 23,24].

The elongated shoots were rooted maximum in half-strength MS basal salts supplemented with 0.25 mg/L IBA with 2% sucrose. At higher concentrations of IBA, the percentage of root induction decrease with an increase in root necrosis and callus formation at the basal end of the shoot. A similar observation was reported by Mony et al. [13]. The percentage of rooting, *number* of roots per explant and days to root

Table 4. Steps of plant regeneration of *Vigna mungo* var. PU30 & PU31

Step		Time (weeks)	Medium
I	*In vitro* shoot development	2-3 weeks	MS basal salts + Vitamins + 1.0 mg/L BA
II	Shoot multiplication	3-4 weeks	MS basal salts + 1.0 mg/L BA
III	Shoot elongation & *in vitro* rooting	2 - 3 weeks	½ strength MS basal salts + 2% (w/v) sucrose + 0.25 mg/L IBA
IV	Primary hardening in the growth chamber	1 week	Sterile distilled water
V	*In vitro* raised plants established in the green house	2-3 weeks	Sand: Soil: Cow-dung (1:1:1)

initiation was significantly varied with different concentrations of IBA. Similar observations were made in *Vigna* species [13], and other leguminous plants *Clitoria ternatea* [12]. Khawar and Özcan [11] reported that MS medium containing 0.25 mg/L IBA performed rooting (25%) within 4 weeks of culture. While, Roy et al. [9] reported that NAA was more effective than IBA for rooting. The well-rooted plantlets were acclimatized in the soil and grew flowering and pod formation. Plant regeneration in legumes depended on cultural factors and source of explants and genotypic differences.

5. CONCLUSION

In conclusion, an attempt was made to develop a rapid propagation system for *Vigna mungo* vars. PU30 and PU31 by manipulating the growth regulators, nutrient salts, and photoperiods. The intensity of shoot morphogenesis was dependent on growth regulators. Though cytokinin rich medium helped in shoot multiplication but 24h photoperiod with 3000 lux light intensity promoted more synthesis of pigment to maintain better growth as well as inhibit the leaf falling from the *in vitro* grown shoots. The pattern of morphogenesis on various phytohormonal regimes largely confirm to other plant species. This protocol will be useful to study the genetic transformation for crop improvement.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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