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In vitro Regeneration of Recalcitrant Green Gram (*Vigna radiata* L. Wilczek) from Immature Cotyledons for Genetic Improvement

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A B S T R A C T

In the present investigation, a simple, rapid and efficient in vitro regeneration system was developed in green gram (*Vigna radiata* L. Wilczek) cv. IPM-02-03 using cotyledonary explants excised from a 4 day old seedling. Organogenic calli were developed from cotyledonary tissues within 4-6 weeks of culture, supplemented with 2.5 mg/L BAP and 1.5 mg/L NAA. Shoot regeneration was achieved from shoot tips and cotyledonary node on MS medium was supplemented with BAP 2.0 mg/l. Regeneration frequency depends on genotype, explants types and growth regulator combinations in the medium. 1.5 mg/l BAP in combination with 1.0 mg/l Kinetin was proved to be the most effective treatment for multiple shoots induction from nodal explants after 3-4 weeks. The number of shoots per culture varied from 1.3 to 7.8 in different growth media. The cultures were incubated for 16 hrs of photoperiod in 25°C for 2 weeks for shoot bud regeneration. The proliferated shoots were further sub cultured on similar medium for higher shoot bud regeneration. Rooting was achieved in all rooting media but elongated shoots were rooted on ½ strength MS media supplemented with 0.5 mg/l NAA or IBA with 2% (W/V) sucrose which produced the maximum number of strong and healthy roots. The rooted plantlets were transferred to soil mixture (Soil: Sand: Vermicompost 1:1:1 ratio) and kept in the green house with 85% humidity. Seventy-five percent plants were successfully grown after regeneration with no phenotypic variation. This protocol can be used for genetic transformation and large-scale production of genetically improved green gram variety.

Keywords

In vitro regeneration, *Vigna radiata*, BAP, NAA, IBA

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Introduction

Green gram (*Vigna radiata* L. Wilczek; family: Fabaceae) is an important nitrogen fixing, short duration pulse crop grown in many parts of India. It is thought to have originated from Indian sub-continent with maximum diversity at Western Ghats (De candole, 1983). It is cultivated most extensively in India, Central Asia, Central Africa, USA and Australia (Pasquet, 2001). It is an important grain legume grown mainly in arid and semi-arid situations across the country during Kharif and Rabi season and

contributes nearly 15% of total production (Yadav *et al.,* 2010). Green gram plays an important role in Indian diet as it contains vegetable protein and also other minerals and vitamins. It supplies easily digestible major share of protein requirement of vegetarian population of the country. The seeds contain 25-28% protein, 1.0-1.5% oil, 3.5-4.5% fiber, 4.5-5.5% ash and 62-65% carbohydrates on dry weight basis (Singh *et al.,* 2016). Sulphur containing amino acids like methionine and cysteine were less in pulses and methionine concentration was more in urd bean and lysine content was more in mung bean. So the protein of mung bean and urd bean are an excellent to rice based diet in terms of balanced human nutrition (Singh *et al.,* 2016).

Though India is the origin of many *Vigna* species but productivity is low due to biotic and abiotic stresses (Dita *et al.,* 2006). Green gram has limited genetic variability for genetic improvement programme. A number of biotic and abiotic stresses are severely affecting the production and productivity of green gram. Novel trait can be incorporated for genetic enhancement by utilizing the trait from wild species of *Vigna*. The gene introgression has already resulted in enhancing the yields and disease resistance. Moreover it facilitates further genetic improvement in green gram. The gene introgression effort have led to the development of improved cultivars of green gram such as IPM-99-125, IPM-02-03 and IPM-02-14 having resistance against YMV and have increased the yield by increasing the grain size and 100 seed weight (Singh *et al.,* 2010). An efficient and successful regeneration and transformation protocol will be the key to genetic improvement of legume but successful regeneration protocol was not well developed as it is a recalcitrant legume and tissue culture was repeatedly described to be difficult (Chandra *et al.,* 2003; Khatun *et al.,* 2008; Eapen, 2008; Sagare *et al.,* 2015). A very little information is available on *in vitro* regeneration of green gram and its reproducibility (Khatun *et al.,* 2008; Sen *et al.,* 1998). Considering the above fact the present investigation was undertaken to develop a simple, efficient and reproducible protocol from nodal explants for *in vitro* regeneration of multiple shoots in green gram.

Materials and Methods

Callus induction and shoot bud regeneration

The seeds of green gram (*V. radiata* L. Wilczek) cv. IPM-02-03 were collected from Centre for Pulse Research, Orissa University of Agriculture and Technology, Berhampur, Ganjam, Odisha (Figure 1a). The healthy and bold seeds were washed with distilled water and then surface sterilized with 70 % ethanol (v/v) for 2 min followed by washing with sterile distilled water for 2–3 times. Seeds were treated with 0.1 % $HgCl_2$ (w/v) solution for 3 min followed by thorough washing for 4 to 5 times with sterile distilled water to remove all the traces of $HgCl_2$ and was blot dried on sterilized filter paper. Sterilized seeds were germinated under aseptic conditions on half-strength MS (Murashige and Skoog, 1962) medium without any growth regulators. The cotyledonary segments (cotyledonary node, hypocotyl) were excised from a four-day old seedling and cultured on modified MS medium containing various concentration of (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/l) BAP or kinetin or indole-3- acetic acid (IAA) or 1-napthaleine acetic acid (NAA) (0.1, 0.5, 1.0, 1.5, 2.0 mg/l) alone or in combination for callus induction and shoot bud regeneration (Table 1).

Fifteen explants were cultured per treatment and each treatment was replicated 3 times. The pH of the medium was adjusted to 5.7 prior to addition of agar (6g/l) and medium was autoclaved at 121°C for 20min. All cultures

were kept at 25± 2°C under white fluorescent light 16 h photoperiod for regeneration. The subculture was made at every 4 weeks interval in the similar medium. The data was taken at every 2–3 weeks interval for regeneration frequency, number of shoot buds per culture and shoot elongation.

Root induction and transplantation

For root induction *in vitro,* the raised shoot (3-4cm long) from MS medium supplemented with 1.0 mg/l BAP were excised and transferred to MS semisolid medium as well as liquid medium fortified with various concentrations of IAA or indole-3-butyric acid (IBA) or NAA (0.1, 0.25 and 0.5 mg/l) alone or in combination of IBA and NAA with 2 % (w/v) sucrose for induction of rooting.

For efficient rooting the cultures were incubated at 25 ± 2 °C in culture room with 16 h photoperiod and 3,000 lux intensity. Various biometrical observations were recorded in terms of average days to bud break, no of shoots per explant. After *in vitro* shooting and rooting, the plants were removed from culture tubes and were washed in running tap water to remove agar from the root. Then the rooted plantlets were kept in distilled water for two days for acclimatization under shed. After two days, the plants were transferred to earthen pots containing soil: sand: vermicompost (1:1:1) mixture for hardening under greenhouse with 85 % humidity. The data was taken at 3 weeks interval with regard to percentage of rooting, days to rooting and types of root.

All the experiments were carried out in the laboratory under completely randomized design (CRD) with three replications. The data were analyzed statistically by F test. F test was carried out after analysis of variance (ANOVA) and CD (P=0.5) and CV % values were calculated where F test was significant.

Results and Discussion

Callus induction

The results of the present investigation revealed high variation in callus induction, shoot and root regeneration. Almost 90-100% nodal explants of green gram cv. IPM-02-03 initiated calli when cultured on modified MS media containing 0.5 mg/l BAP (Table 1). Profuse callus were induced regardless of explants used (Figure 1b). Media devoid of BAP failed to induce callus from cotyledonary nodes. It reveals that for induction of callus from cotyledonary nodes, cytokinin might be indispensible (Khatun *et al.,* 2008). Culturing of explants on MS medium initially gave rise to creamish green callus on media containing BAP alone and nodular greenish callus was obtained when BAP was used in combination with NAA (Table 1, Fig. 1b). The callusing frequency ranged from 60-90% which seemed to depend more on concentration of BAP. The callus diameter was higher on medium containing higher concentration of BAP along with NAA and IAA (Table 1). MS media supplemented with 3.0 mg/l BAP along with NAA 0.5 mg/l have produced 92% of callus from explants. MS media supplemented with 2.5 mg/l BAP along with 0.5 mg/l NAA had produced better callus having the highest diameter. Highest shoot bud regeneration from callus was found on MS media containing 2.5 mg/l BAP. The problem of browning developed mostly in nodal explants and epicotyls derived calli. Excessive browning and root formation was observed in 4 weeks old callus culture which did not undergo subculture. Prolonged culture for 5 weeks caused severe browning and eventually death of calli. This is possibly due to accumulation of phenolic compound (Avenido *et al.,* 1990). Culture medium without growth regulators did not show any positive effect on organogenic callus formation as well as shoot bud regeneration (Table 1).

Direct shoot and root induction

In general *in vitro* regeneration of *Vigna* sp is more limited than other grain legume. In Vigna *radiata* L. the plants are regenerated directly from explants e.g., shoot tips, cotyledonary node, primary leaf and hypocotyl. Shoot bud regeneration from cotyledonary tissues of green gram were regenerated on MS medium supplemented with various concentrations of BAP or Kinetin supplemented with NAA or IAA (Fig. 1c, 1d). There was no formation of callus and shoot bud without growth regulator in MS medium. Among the two cytokinins tested, kinetin along with BAP showed higher rate of shoot bud regeneration (Fig. 1d). Similar results have been reported by (Varalaxmi *et al.,* 2007). Auxin supplementation in the culture media enhanced the shoot bud regeneration. BAP in combination with NAA also enhanced the higher frequency of regeneration as compared to BAP with IBA which supported the result of Adlinge *et al.,* (2014). Shoot regeneration was increased with increase in BAP concentration but with BAP concentrations more than 4.0 mg/l in the culture medium, the rate of regeneration was reduced. The maximum frequency of regeneration (72.4 %) was achieved on MS medium supplemented with 2.0 mg/l BAP and 1.5 mg/l NAA after 6 weeks of culture (Table 1; Fig. 1c, 1d). Adenyl sulphate along with BAP and kinetin showed higher rate of shoot bud elongation and regeneration (Fig. 1d).

Table.1 Effect of various concentrations of auxin and cytokinin in callus development

Growth regulators			Responding Explant (%)(mean ± SE)	Callus Diameter (mm) (mean ± SE)	Regeneration Frequency (%)(mean ± SE)	Number of shoots Per explants (mean ± SE)
BAP (mg/l)	NAA (mg/l)	IAA (mg/l)				
0	0	0	n.d	n.d	n.d	n.d
0.5	0	0	75±2.11	4.6±0.58	48.6±3.23	0.84±0.26
1.0	0	0	80±3.41	5.1±0.33	54.4±3.54	1.22±0.45
1.5	0	0	82±3.62	5.4±0.61	61.1±3.84	2.6±0.32
2.0	0	0	88±4.56	5.9±0.43	68.2±2.96	3.8±0.51
2.5	**0**	**0**	**90±5.62**	**6.5±1.23**	**83.4±3.33**	**4.3±0.43**
3.0	0	0	85±4.21	6.7±1.25	72.1±2.13	3.1±0.31
0.5	0.5	0	70±5.12	6.5±1.69	58.8±1.94	1.3±0.22
1.0	0.5	0	74±4.86	6.9±1.87	52.4±3.15	2.1±0.29
1.5	0.5	0	76±3.61	7.2±2.15	74.3±3.26	3.8±0.46
2.0	0.5	0	83±3.46	7.6±1.22	64.6±2.33	3.6±0.38
2.5	0.5	0	86±4.12	8.7±1.56	79.8±3.33	4.1±0.54
3.0	0.5	0	92±4.48	8.2±1.56	82±3.12	4.4±0.52
0.5	0	1.0	67±2.16	6.8±0.33	n.d	n.d
1.0	0	1.0	72±2.48	6.4±0.53	n.d	n.d
1.5	0	1.0	76±3.12	6.9±0.46	n.d	n.d
2.0	0	1.0	83±3.51	7.1±1.26	n.d	n.d
2.5	0	1.0	89±4.56	7.9±1.56	n.d	n.d
3.0	0	1.0	84±4.21	7.6±1.85	n.d	n.d
CD(p=0.05)			3.78	0.64	3.42	0.18

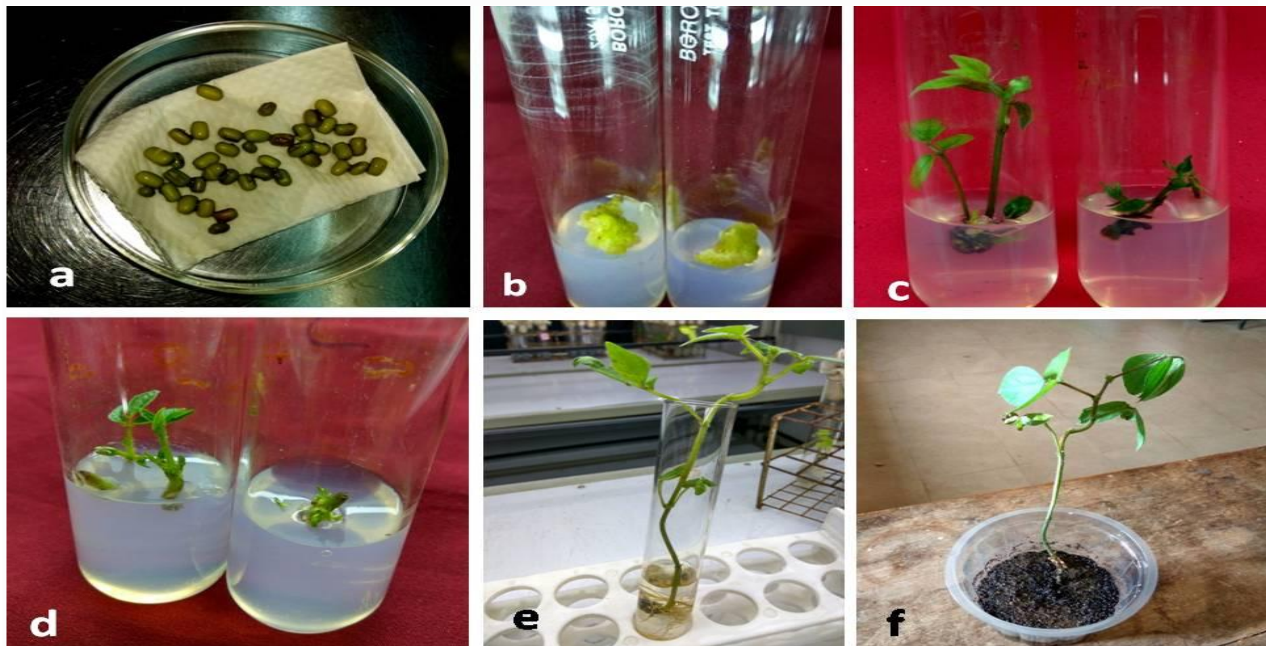
Table.2 Effect of plant growth regulators (PGRs) on shoot bud regeneration from cotyledon explants of *V. radiata* var. IPM-02-03 after sub-culturing

MS + growth regulators (mg/l)					% of shoot bud regeneration (mean ± SE)	No. of shoots/culture (mean ± SE)	Days required to shoot induction (mean ± SE)	Shoot length (cm) after 6 weeks of culture (mean ± SE)
Kn (mg/l)	**BAP (mg/l)**	**NAA (mg/l)**	**IAA (mg/l)**	**Adenine sulfate (mg/l)**				
0.0	0.0	0.0	0.0	0.0	0.0	0.0	00	00
0.5	0.0	0.0	0.0	0.0	0.0	0.0	00	00
1.0	0.0	0.0	0.0	0.0	11.5 ± 0.8	1.50 ± 0.5	32.5±0.5	0.75±0.3
1.5	0.0	0.0	0.0	0.0	18.2 ± 1.2	1.25 ± 0.6	31.6±0.4	2.30±0.23
2.0	0.0	0.0	0.0	0.0	22.8 ± 1.4	2.75 ± 0.7	29.4±0.6	2.50±0.18
3.0	0.0	0.0	0.0	0.0	16.2 ± 1.2	1.88 ± 0.4	28.1±0.9	2.62±0.41
4.0	0.0	0.0	0.0	0.0	8.4 ± 0.76	1.12 ± 0.5	34.4±1.2	1.60±0.26
2.0	0.0	1.0	0.0	0.0	32.4 ± 1.0	2.76 ± 0.5	27.5±0.93	1.92±0.35
2.0	0.0	1.5	0.0	0.0	34.5 ± 1.2	2.25 ± 0.5	28.4±0.7	2.9±0.21
3.0	0.0	1.5	0.0	0.0	17.6 ± 1.4	2.56 ± 0.3	36.4±0.6	2.6±0.67
0.0	1.0	0.0	0.0	0.0	14.6 ± 0.9	2.13 ± 0.2	34.68±0.9	3.4±0.46
0.0	2.0	0.0	0.0	0.0	34.2 ± 1.1	2.45 ± 0.5	28.3±0.72	3.5±0.43
0.0	3.0	0.0	0.0	0.0	42.8 ± 1.3	2.56 ± 0.3	30.3±0.6	2.9±0.72
0.0	4.0	0.0	0.0	0.0	36.7 ± 0.9	1.81 ± 0.4	31.4±0.82	2.4±0.44
0.0	2.0	1.5	0.0	0.0	68.3 ± 1.4	3.32 ± 0.8	28.4±0.91	3.9±0.26
0.0	3.0	1.5	0.0	0.0	66.6 ± 1.2	3.54 ± 0.9	29.6±0.6	3.7±0.48
0.0	2.0	2.0	0.0	0.0	62.8 ± 1.3	5.85 ± 0.9	17.8±0.41	3.1±0.29
0.0	3.0	2.0	0.0	0.0	54.4 ± 1.3[b]	4.21 ± 0.8	18.6±0.64	3.2±0.34
0.0	**2.0**	**1.5**	**0.0**	**25.0**	**72.4 ± 1.0**	**7.8 ± 0.82**	**15.26±0.9**	**4.75±0.25**
0.0	2.0	1.5	0.0	50.0	67.8 ± 0.9	6.32 ± 0.9	17.8±0.6	3.9±0.35
0.0	3.0	1.5	0.0	25.0	64.5 ± 1.2	6.16 ± 0.8	31.4±0.8	3.5±0.51
0.0	3.0	1.5	0.0	50.0	66.8 ± 1.5	6.38 ± 1.0	33.2±0.6	2.6±0.24
0.0	2.0	0.0	1.5	0.0	41.5 ± 0.8	3.82 ± 0.6	38.1±1.1	1.46±0.33
0.0	2.0	0.0	2.0	0.0	45.4 ± 1.3	2.88 ± 0.7	36.4±1.2	1.35±0.33
CD(p=0.05)					4.23	0.18	0.28	0.26

Table.3 Effect of different concentrations of auxins on root induction from excised shoots of *V. radiata* var. IPM-02-03

½ MS + growth regulators (mg/l)			Days to rooting	% of rooting (mean ± SE)	Types of root	Root length (cm) (mean ± SE)
IAA (mg/l)	**IBA (mg/l)**	**NAA (mg/l)**				
0.0	0.0	0.0	0.0	0.0	–	0.0
0.1	0.0	0.0	11–12	25.8 ± 1.2	Short, thick	0.56±0.05
0.25	0.0	0.0	11–12	30.2 ± 1.6	Short, thin	0.84±0.04
0.50	0.0	0.0	13–14	32.8 ± 1.3 b	Short, thick, single	1.96±0.03
0.0	0.1	0.0	9.0	46.2 ± 2.5	Short, thin	2.46±0.05
0.0	0.25	0.0	9–10	56.8 ± 2.6	Long, thin,	2.94±0.05
0.0	0.50	0.0	9–10	62.4 ± 3.2	Long, thin, single	3.45±0.04
0.0	0.0	0.10	8–9	67.2 ± 3.4	Long, thin,	3.65±0.07
0.0	0.0	0.25	8–9	76.6 ± 2.8	Long, thin, multiple branching	4.32±0.06
0.0	0.0	0.50	8–9	56.4 ± 3.2	Long, thin, branching	1.26±0.02
0.0	0.1	0.25	9–10	64.4 ± 2.7	Long, thin, branching	1.38±0.02
CD(p=0.05)			0.45	3.27		0.17

Fig.1



a. Seeds of green gram (*Vigna radiata* L. Wilczek) cv. IPM-02-03.
b. Development of callus from cotyledonary node of *V. radiata* grown on MS medium supplemented with 2.0 mg/l BAP and 1.5 mg/l NAA after 4 weeks of subculture.
c. Development of shoot buds from cotyledon derived callus of *V. radiata* grown on MS medium supplemented with 2.0 mg/l BAP and 1.5 mg/l NAA after 8 weeks of subculture.
d. Direct development of shoot buds from cotyledonary node of *V. radiata* without callus phase grown on MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA after 8 weeks of subculture.
e. Proliferation of roots from excised shoots of *V. radiata* grown on MS medium supplemented with 0.25 mg/l NAA after 8 days of culture.
f. Rooted plantlets kept in sterile distilled water before transfer to pot.
f. Rooted plantlets establish in soil with normal growth before transplanted in field.

The maximum multiple shoot production was obtained from cotyledonary node when cultured in MS media fortified with 1.5 mg/l BAP and 1.0 mg/l kinetin. Shoot multiplication and propagation of green gram have also been reported by different workers using different explants (Sagare *et al.,* 2015; Khatun *et al.,* 2008; Vijayan *et al.,* 2006; Gulati *et al.,* 1990). Increased BAP concentration from 2.0 to 3.0 mg/l, the rate of shoot bud regeneration increased but the shoot growth was reduced.

Higher concentration more than 4.0 mg/l BAP and kinetin more than 2.0 mg/l showed reduced shoot bud regeneration. It was observed that BAP enhances regeneration frequency irrespective of the basal medium and nature of explants and similar findings were also reported by Gulati and Jaiwal (1990); Yadav *et al.,* (2010) and Shiv Kumar (2010).

The cultures were initially incubated for 2 weeks in dark and subsequently transferred to 16 h photoperiod, which showed higher rate of shoot multiplication as compared with the cultures incubated in completely 16 h photoperiod. The cytokinin has significant effect on shoot multiplication as reported earlier in other legume crops like chickpea and black gram (Yadav *et al.,* 2010; Mony *et al.,* 2010; Das *et al.,* 2002). In the present study, the maximum (7.80) number of shoot buds/culture was obtained within weeks culture on MS medium supplemented with 2 mg/l BAP + 1.5 mg/l NAA + 25 mg/l Ads (Table 2). Muruganantham *et al.,* (2005) reported that the multiple shoot induction (6 shoot buds/explant) was obtained from axillary shoots on medium having 1.0 mg/l BAP. Mony *et al.,* (2010) reported that the lowering of BAP concentration (1.0 mg/l) produced maximum number of shoots (9.33) in case of black gram also stated that the increased BAP concentration in medium resulted callus formation. Well-developed rooting system is necessary for better establishment of the plantlet in the field. Healthy shoots were cultured on different root induction media (half strength MS medium supplemented with IAA or IBA or NAA) alone as well as in combination for root induction. Root initiation was not achieved on medium without growth regulators. Root initiation developed in half-strength MS medium supplemented with 0.1–0.25 mg/l NAA or IBA after 8 to 10 days of culture (Fig. 1e). The medium having NAA showed better root formation as compared with IBA (Table 3). The medium having both IBA and NAA resulted in low percentage of rooting and formation of globular callus at the cut end. The percentage of rooting varied from 25.8 to 76.6 % depending on growth regulators used. The roots were branched, thin and elongated. However, IBA was effective for root induction as reported by Das *et al.,* (2002), Geetha *et al.,* (1998), Mony *et al.,* (2010) found that percentage of rooting was higher in IBA but number of roots per plant was higher in NAA.

Transplantation in soil

The rooted plantlets were transferred to distilled water and kept for 72 h for acclimatization (Fig. 1e). Further, the plantlets were transferred to polythene bag containing soil: sand: vermi-compost (1:1:1) and kept in the greenhouse for 15 days with 85 % relative humidity (RH). After 2 weeks, these plantlets were transferred to poly-house for hardening and final establishment (Fig. 1f). Survival percentage of the transplanted plantlets was 70–75 %. Further, the plantlets grew normally.

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