An efficient *in vitro* regeneration protocol to generate stable transgenic lines of black gram (*Vigna mungo* L.)

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Abstracts
An efficient and quick *in vitro* regeneration protocol was developed for black gram (*Vigna mungo* L.) using wounded embryonic axis with cotyledon as explant. Murashige and Skoog (MS) medium supplemented with 4.44 µM BAP and 2.32 µM Kinetin was found to be effective in producing maximum number (mean 7.80) of multiple shoots. The individual shoots elongated to 4.5 cm when MS medium was supplemented with 2.89 µM GA3 along with 0.44 µM BAP and 0.46 µM KIN. A novel *in vitro* rooting technique was also optimized for black gram using half-strength liquid MS medium supplemented with 1.34 µM NAA. The shoots in this medium produced the highest number (mean 7.50) of roots with root length of 6.02 cm. The plantlets were transferred to soil mixture and placed in greenhouse where more than 80% successfully grew to maturity. The same protocol was successfully used to generate transgenic black gram lines carrying Bt-Cry2Aa gene through *Agrobacterium*-mediated transformation with a transformation efficiency of 0.42%. The rooted *T₀* plants grew to maturity and produced *T₁* seeds with the presence and expression of transgene in *T₁* plants. Thus, we have standardized an *in vitro* regeneration protocol suitable for generation of stable transgenic black gram plants.

Key words: Black gram, *Vigna mungo*, *in vitro* regeneration, *Bacillus thuringiensis*, Cry2Aa

Introduction
Black gram (*Vigna mungo* (L.) Hepper) is an important grain legume which serves as a main source of dietary protein for the majority of population in developing countries of Asia, Africa and Latin America (Eapen and George 1990). However, productivity of black gram is low, which is primarily attributed to abiotic and biotic stress like drought, weeds, insect pests and diseases. Among these, insect pests cause a serious threat to black gram production by increasing cost of cultivation and impairing quality of produce resulting in yield loss up to 55.20% (Vikrant et al. 2015). Conventional breeding to overcome such stress is not feasible due to narrow genetic diversity. Therefore, an efficient regeneration system is required for introduction of Cry2Aa genes in black gram against insect pests. *In vitro* regeneration has previously been reported in black gram using various explants either through indirect shoot organogenesis (Sen and Mukherjee 1998; Das et al. 1998; Ignacimuthu and Franklin 1999; Das et al. 2002; Varalaxmi et al. 2007; Adlinge et al. 2014) or directly from immature cotyledonary nodes (Muruganatham et al. 2005), embryonic axes (Acharjee et al. 2012) and leaf petiole explants (Sainger et al. 2015). However, black gram, like other grain legumes, is recalcitrant to regeneration and transformation and the reported protocols are not reproducible and/or cultivar dependent. Most of the published protocols used TDZ for inducing proliferation of greater number of shoots per explant but with attendant disadvantages like stunted growth and low efficiency of rooting. Another major constraint in *in vitro* regeneration of grain legumes like chickpea, cowpea, pigeon pea, black gram etc., is the induction and development of a strong root system due to which the success rate of establishment of the whole plant in the greenhouse is very low. The response of rooting also depends on the nature of medium (liquid or solid) used (Romero et al. 1998). The liquid medium offers advantages over semi-solid medium in increasing the rooting frequency up to 90% (Jayanand et al. 2003) and facilitates recovery of a larger number of rooted plants during hardening resulting quick and easy transfer to soil.

The successful passage of transformed shoots through several stages of selection is very critical. The recovery of *in vitro* selected transformed shoots...
as a result of *Agrobacterium* infection after several cycles of selection due to the presence of the selectable marker gene is very low and because of low frequency of rooting of the transformed shoots, most of the plants do not survive during the hardening procedure. Genetic transformation studies in black gram have been limited to only using *uidA* and *nptII* gene as reported earlier by Saini et al. (2002), Saini and Jaiwal (2005, 2007), Acharjee et al. (2012) and Sainger et al. (2015).

In the present investigation an efficient *in vitro* regeneration of black gram shoots in MS medium containing only growth regulators without using TDZ to minimize the obstacles that result in response to TDZ and a liquid root induction medium was optimized for the generation of transgenic black gram plants. This is the first report of introgression of Bt gene into the black gram plants using an *Agrobacterium* *ium* mediated genetic transformation system.

**Materials and methods**

**Plant material media and culture conditions**

The seeds of *Vigna mungo* (L.) Hepper collected from the local market was used for tissue culture experiments. Seeds were stored in plastic containers at 4°C. The explants were inoculated in the shoot regeneration medium I (SRI), containing Murashige and Skoog (MS) (1962) macronutrients, micronutrients, vitamins, 3% sucrose, 0.8% agar and various concentrations of BAP (2.22, 4.44, 6.66, 8.88, 11.10 and 13.32 µM), Kinetin (2,3, 4.65, 6.90 and 9.30 µM) and NAA (0.26 µM). The shoot regeneration medium II (SRII) comprised of all the components of SRI medium except NAA. The shoot elongation (SE) medium was composed of BAP (0.44 µM) and KIN (0.46 µM) (Table 1). Different concentrations of GA3 (1.44 and 2.89 µM) were also added to the SE medium (Table 2). The pH of the media was adjusted to 5.8 prior to autoclaving. All the media were autoclaved at 121°C for 20 min at 1.05 kg/cm² pressure. Cultures were maintained under white fluorescent light at a photon flux of 24 µM m⁻² s⁻¹ for 16/8 h light/dark photoperiod at 25±2°C. About 20 g of seeds was taken and surface sterilized under laminar air flow cabinet. The seeds were treated with 70% ethanol for 1-2 min and kept in 10% sodium hypochlorite for 20 min. The seeds were further washed with sterile water for three to four times to remove excess surfactants and soaked overnight in sterile water.

**Explant preparation and in-vitro regeneration**

The seeds were bisected longitudinally along the emerging radicals using a sterilized surgical blade into two halves to essentially create a wound in the embryonic axes. The wounded embryonic axis with cotyledon was selected as explant (Fig. 2A). All explants were pretreated in liquid MS basal medium for 30 min to prevent the wounded axes from oxidation.

After surface sterilization, the explants (wounded embryonic axes with cotyledons) were placed into 100 x 15 mm Petri dishes containing SRII medium. After 12-14 days, the regenerating explants were subcultured in SRII medium. An individual experiment was carried out in triple for each treatment with 20 explants in SRII medium. A total of 10 explants were placed per plate. The number of multiple shoots produced per explant was recorded after 14-16 days of subculture of the explants on to SRII medium. Multiple shoots produced were separated into individual shoots and sub-cultured in SE medium supplemented with GA3. The shoots that elongated to 4-5 cm were transferred to rooting media.

The elongated shoots were rooted in different root induction media containing half-strength MS medium supplemented with different concentrations of NAA (1.34 and 2.68 µM), IBA (1.23 and 2.46 µM) or IAA (1.42 and 2.85 µM). The experiments were carried out in triplet with 10 individual healthy shoots rooted in different rooting media. The rooting media were either liquid or solidified with 0.6% Agar. The number

<table>
<thead>
<tr>
<th>Media</th>
<th>MS + Growth regulators (µM-1)</th>
<th>Per cent of shoot bud regeneration</th>
<th>Mean no. of healthy shoots/explant (mean±SE)</th>
</tr>
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<tbody>
<tr>
<td>SRI.1</td>
<td>0.00 0.00</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SRI.2</td>
<td>2.22 2.32</td>
<td>64.86</td>
<td>4.75±0.14b</td>
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<tr>
<td>SRI.3</td>
<td>4.44 2.32</td>
<td>80.00</td>
<td>7.80±0.10a</td>
</tr>
<tr>
<td>SRI.4</td>
<td>2.22 4.65</td>
<td>93.61</td>
<td>3.84±0.66c</td>
</tr>
<tr>
<td>SRI.5</td>
<td>4.44 6.97</td>
<td>66.00</td>
<td>3.60±1.03c</td>
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<tr>
<td>SRI.6</td>
<td>6.66 4.65</td>
<td>62.66</td>
<td>3.54±0.43cd</td>
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<tr>
<td>SRI.7</td>
<td>8.88 6.97</td>
<td>54.28</td>
<td>3.20±0.19cd</td>
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<tr>
<td>SRI.8</td>
<td>6.66 9.00</td>
<td>53.33</td>
<td>2.50±0.56cd</td>
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<tr>
<td>SRI.9</td>
<td>11.10 6.97</td>
<td>55.17</td>
<td>2.50±0.58c</td>
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<tr>
<td>SRI.10</td>
<td>13.32 6.97</td>
<td>52.17</td>
<td>1.60±0.39e</td>
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*Means with the same alphabet in a column are not significantly different according to Duncan’s multiple range test at 5% level
**Values are means of three replicates with 20 explants in each replication.
of roots produced per shoot and its root length were recorded after 10-12 days of inoculation in rooting media. The growth regulators, IBA and IAA were filter sterilized and added to the autoclaved media prior to pouring. However, NAA was added to the MS media and then autoclaved. All culture conditions remained the same as described above for in vitro regeneration.

**Hardening and transferring of the rooted plants**

The hardening process involved three stages viz., initial transfer of plants into small pots containing soil mixture with covers, acclimatization phase during which plants were gradually exposed to sunlight and finally the plants were transferred to greenhouse. The shoots that produced roots in various root induction media were carefully taken out of the culture tube and washed carefully in running tap water. The rooted shoots were transferred to small pots and covered with transparent polypropylene bags for 7-10 days. The moisture inside the bags was wiped with tissue paper every day. When new shoots developed, the cover was removed completely and after 10 days the plants were transferred to bigger pots and finally to the greenhouse. A total of 10 rooted shoots from each root induction media were carried out in triplets for the hardening procedure.

**Agrobacterium-mediated transformation**

The disarmed Agrobacterium strain (Agl1) harboring
the binary vector pBIN-AR, which contains the Cry2Aa gene driven by CaMV35S promoter and a neomycin phosphotransferase gene (nptII) driven by nosA promoter (Fig. 5A) was used for transformation studies. The Agrobacterium culture was grown in MGL medium (Garfinkel et al. 1980) in presence of kanamycin (50 mg/L) at 28°C overnight in an environmental shaker. The explants were incubated with the bacterial culture for 45 minutes to 1 h for infection. After infection, the explants were inoculated in B5 medium (Gamborg et al. 1968) for 3 days. The same in vitro regeneration protocol was followed for the genetic transformation of black gram. Selections of the explants were done at 200 mg/L kanamycin monosulphate.

**Confirmation of Cry2Aa gene in putative transgenic plants**

Total genomic DNA was extracted from fresh leaves of putative transformants (T₀) and non-transformed (control) plants by the CTAB (cetyl trimethyl ammonium bromide) method (Rogers and Bendich 1988). Putative transgenic (T₀) plants and T₁ progeny were screened by PCR for the presence of the Cry2Aa gene. A 680 bp size of amplicon was amplified using forward (F1: GGTGGAGAGGCTATGTCCTGA) and reverse (R1: GGTAGCCAAACGTATGTCCCTGA) primers for the coding region of Cry2Aa gene. 200 ng/µl of DNA was used for PCR reaction in 1X reaction buffer containing 3mM MgCl₂, 120 µM of each dNTP, 2 pmole of each primer and 1 unit of Taq DNA polymerase. The PCR conditions used were initial denaturation at 94°C for 10 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min followed by a final extension at 72°C for 7 min. The genomic DNA from untransformed control plants and Cry2Aa positive chickpea transgenic lines (72C2) were used as negative and positive controls, respectively. Products were resolved on 1.0% agarose gel stained with Ethidium Bromide (EtBr) in 1X TBE buffer. Images of gel were documented in Gel Documentation system. Segregation analysis was carried out based on PCR analysis using Cry2Aa primers on the progeny of three transformants (VM1, VM2 and VM3).

**Estimation of Cry2Aa protein in transgenic black gram plants**

Western blot was prepared following the protocol described by Sarmah et al. (2004) for the T₁ progeny for the expression of the Cry2Aa protein. Presence of 66 KDa band confirmed the expression of the Cry2Aa protein. For western hybridization, total protein was isolated from young black gram leaves using protein extraction buffer (0.1M Tris pH 8.0, 0.5M NaCl, 1mM EDTA and 50µM BME) as described by Sarmah et al. 2004. Quantification of protein was performed using Bradford reagent (Sigma Aldrich, United States) (Bradford, 1976) and 40 µg protein was fractionated on 15% acrylamide gel with 10% SDS and blotted on to nitrocellulose membrane (Amersham™ Protran® Western blotting membranes, nitrocellullose, United States) by wet transfer. Cry2Aa specific primary antibody (Amar Immunodiagnostics Pvt. Ltd., Hyderabad, India) was used and horse radish peroxidase conjugated secondary antibodies (Promega, India) were used for detection. The blot was developed using a BCIP (5-bromo-4-chloro-3'-indolylphosphate p-toluidine) substrate (Sigma Aldrich, United States).

**Statistical analysis**

The results of experiments for multiple shoot induction, effect of GA3, rooting and treatments with different growth regulators were tested by ANOVA. The differences of means for numbers of multiple shoots, effects of GA3 on shoot elongation and the number of roots per explants were tested by Duncan’s Multiple Range Test (Duncan 1955).

**Results**

**In vitro regeneration**

Shoot buds were observed at the end of 14 days in the first shoot regeneration (SRI) media (Fig. 3B). Multiple shoots were observed in the second shoot regeneration (SRII) media at the end of 14 days (Fig. 3C). The number of multiple shoots produced was recorded during this stage. The effect of various concentrations of BAP, Kinetin and NAA on induction of multiple shoot from the embryonic axis is presented in Table 1. The highest number of multiple shoots (mean 7.80) was observed in SRI.3 medium (4.44 µM BAP, 2.32 µM Kinetin and 0.26 µM NAA) followed by SRII.3 medium (4.44 µM BAP and 2.32 µM Kinetin) (Fig. 3C). Though the number of multiple shoots was highest in this medium, the maximum frequency of induction of multiple shoots per explant was observed in SRI.4 medium as compared to SRI.3 medium. The hormone-free MS medium produced a single elongated shoot. Higher concentration of BAP (13.32 µM) and Kinetin (6.97 µM) resulted in increased diameter of the single shoot. However, the MS media supplemented with BAP (8.88 or 11.10 µM) and Kinetin (4.65, 6.97 or 9.30 µM) produced only 3-4 multiple shoots per explant.
The hormone GA3 was used at two concentrations (1.44 µM or 2.89 µM) in the shoot elongation (SE) medium to see the effect of GA3 on elongation of the regenerated shoots. GA3 at 2.89 µM showed elongation of shoots to 4.5 cm at the end of 20 days (Fig. 3D). However, in shoot elongation medium without GA3 or with 1.44 µM GA3, the shoot length increased to only 1 cm at the end of 20 days.

The formation of roots on elongated shoot was observed within 1-2 weeks. Early rooting was observed within 7-10 days in the liquid half-strength MS rooting medium, however, it took 12-15 days in semi-solid half-strength MS rooting medium supplemented with different auxins. The percentage of shoots producing roots varied from 20% to 90% (Table 2). Rooting of in vitro regenerated black gram shoot was observed in all tested half-strength MS media supplemented with different auxins (Fig. 4). The highest percentage of independent shoot producing roots was recorded in the half-strength MS liquid medium supplemented with 1.34 µM NAA. The mean number of roots per shoot was found to be 7.50 and mean root length was recorded to be 6.02 cm (Fig. 4H). The rooted plants were successfully established in the greenhouse when roots were induced in half-strength MS liquid medium supplemented with 1.34 µM NAA and were grown to maturity. A schematic representation of the in vitro regeneration of black gram has been summarized in Fig. 2.

**Agrobacterium-mediated genetic transformation**

A total of 3500 explants were infected and co-cultivated with Agrobacterium strain carrying the binary plasmid pBIN-AR. In vitro shoots (total 18) survived several rounds of kanamycin selection and were transferred to soil in greenhouse. Out of 18 plants tested, 15 were PCR-positive for Cry2Aa gene showing a transformation frequency of 0.42%. However, only nine PCR-positive plants grew till maturity (Fig. 5B).

**Segregation of Cry2Aa gene in T1 progeny**

The progeny of three transgenic plants (VM1, VM2 and VM3) were screened by PCR analysis for the transmission of Cry2Aa gene. In the line VM1, out of 18 T1 progeny, 16 were found to be positive and 2 were negative for the presence of transgene. In the case of line VM2, out of 11, 6 were positive and 5 were negative for transgene amplification. The number of positive segregants was 12 and number of negative segregants was 2 in the case of line VM3. This indicates the transmission of transgene in to T1 generation in all these three lines. Though, X2 analysis (not presented), confirmed segregation of transgene as 3:1 in the line VM2 and VM3 and 15:1 in the case of line VM1; further confirmation can be made only after analyzing more numbers of progeny in the next generation.

**Expression of Cry2Aa protein in the leaves of T1 progeny**

The western blot analysis confirmed the expression of Cry2Aa protein in the T1 progeny of the lines VM1, VM2 and VM3. The presence of a 66 KDa polypeptide of Bt protein on the blot confirmed the expression of Cry2Aa protein in the T1 progeny of the line VM3 (Fig. 5C).

**Discussion**

In Agrobacterium mediated transformation system, the T-DNA transfer takes place near the wounded tissues (Gelvin 2003). Therefore, explants were bisected longitudinally in order to create a wound. To enhance the production of multiple shoots from the site of injury, the tip of the shoot apex was removed from the explant to suppress the growth of apical meristem. De novo organogenesis triggered adventitious shoots from the base of the wounded embryonic axis with attached cotyledon as reported earlier by Acharjee et al. (2012). The hormone cytokinin plays an important role in the multiplication of shoots in many legume crops like chickpea and mungbean (Gulati and Jaiwal 1990; Polisetty et al. 1997; Vijayan et al. 2006; Yadav et al. 2010). BAP alone cannot induce large number of multiple shoots in any of the explants like cotyledon, hypocotyls and excised cotyledons as discussed by Adlinge et al. (2014). To increase the number of multiple shoots, besides BAP, Kinetin and NAA, several authors used supplements of cytokinins like TDZ, Zeatin, amino acids like proline and Adine Sulphate (Das et al. 1998; Sen and Mukherjee 1998; Ignacimuthu and Franklin 1999; Das et al. 2002; Muruganatham et al. 2005; Varalaxmi et al. 2007; Acharjee et al. 2012; Adlinge et al. 2014). Though the mechanism of TDZ is not known, it does increase the number of multiple shoots. Thomas and Katterman (1986) described that TDZ leads to differentiation of plantlets by increasing the biosynthesis and accumulation of cytokinin. However, there are certain disadvantages of using TDZ, such as stunted shoots and abnormal leaf morphology. Therefore, in the present study no supplements like TDZ or adenine sulphate were used in the shoot induction media. High
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Fig. 2. (A) Explant (wounded embryonic axis with cotyledon). (B) Shoot regeneration from the wounded embryonic axis with cotyledon

Fig. 3. A Schematic representation of in vitro regeneration of Vigna mungo from wounded embryonic axis with cotyledons. A = Explant, cotyledon with wounded embryonic axis; B = Shoots regenerated in first shoot regeneration (SRI) medium after 12 days of inoculation; C = Multiple shoots regenerated in second shoot regeneration (SRII) medium after 14-16 days of inoculation; D = Shoots elongated in Shoot Elongation (SE) medium; E = In vitro shoots producing healthy and elongated shoots in half-strength liquid MS medium supplemented with 1.34 µM NAA; F = A mature plant established in greenhouse

Fig. 4. In vitro shoots produced roots in different rooting medium (A-F) Solid half-strength MS media supplemented with A = 2.68 µM NAA; B = 1.34 µM NAA; C = 2.46 µM IBA; D = 1.23 µM IBA, E = 2.85 µM IAA; F = 1.42 µM IAA and (G-K) Liquid half-strength MS media supplemented with G = 2.68 µM NAA; H = 1.34 µM NAA; I = 2.46 µM IBA; J = 1.23 µM IBA, K = 2.85 µM IAA and L = 1.42 µM IAA

Fig. 5. A = Schematic representation of the Cry2Aa gene construct in the pBIN-AR binary vector. The Cry2Aa gene is driven by a constitutive 35S promoter. B = PCR analysis of T0 transgenic plants of Vigna mungo with Cry2Aa gene specific primers, M = DNA ladder; P = a 720 bp amplicon of genomic DNA of positive chickpea transgenic line, 72C2; N = Negative control (DNA from untransformed blackgram plant); W = Internal Control (Water); VM1, VM2 and VM3 (T0 transgenic plants). C = Western blot analysis for expression of Cry2Aa protein in the T1 transgenic lines of VM3. M = pre-stained protein ladder; P = Protein from Cry2Aa positive chickpea transgenic line, 72C2; N = Protein from untransformed black gram plants; 1-6 (T1 transgenic plants of VM3)
concentration of BAP resulted in callus formation as reported earlier (Ignacimuthu et al. 1997; Varalaxmi et al. 2007; Muruganatham et al. 2005; Shariful et al. 2010 and Adlinge et al. 2014). In the present investigation, when MS medium was supplemented with 2.89 \( \mu \text{M} \) (1mg/L) GA3 along with 0.44 \( \mu \text{M} \) (0.1 mg/L) BAP and 0.46 \( \mu \text{M} \) (0.1 mg/L) Kinetin, individual shoots elongated to an average length of 4.5 cm. However, there was no significant increase in shoot length when GA3 was added or not at a concentration of 1.44 \( \mu \text{M} \) (0.5 mg/L). Muruganatham et al. (2005) reported that 0.6 mg/L GA3 promoted maximum shoot elongation in black gram.

Prolonged incubation of shoots in root induction media resulted in the ageing which had a negative effect on normal growth and seed yield (Ghosh et al. 2017). Though there are reports of various direct rooting protocols in black gram (Saini et al. 2002; Muruganatham et al. 2005; Harisaranraj 2008; Adlinge et al. 2014), those protocols lack detailed information about the number of roots produced per explant and root length. Direct rooting protocol in solidified MS media supplemented with IBA, IAA and NAA was tried in black gram but most of the protocols required at least 12-14 days for obtaining well developed healthy elongated roots with low success rate in plant establishment in the soil during the hardening procedure (Gill et al. 1987; Ignacimuthu et al. 1997; Eapen et al. 1998; Ignacimuthu and Franklin 1999; Franklin et al. 2000; Tyagi et al. 2001; Agnihotri et al. 2001; Villiers et al. 2008; Shariful et al. 2010; Adlinge et al. 2014). In the present study, rooting was observed in all tested semi-solid and liquid half-strength MS media supplemented with different auxins (IBA, NAA and IAA) at various concentrations. The liquid medium used in the present study was suitable for increasing the frequency of establishment of the transgenic plants in the greenhouse which might be due to minimum exposure of \textit{in vitro} shoots in the liquid medium wherein less number of days required for the root initiation. This rooting procedure took 7-12 days for the roots to develop compared to the other solidified half-strength MS media supplemented with different growth regulators wherein 12-14 days required for root initiation. The roots were healthy and elongated to an average of 7 cm. One of the main advantages of using liquid medium is that it increases the frequency of plant establishment in greenhouse. However, one has to be very careful while selecting the shoots for the rooting medium. Healthy shoots with multiple leaves primordia were found to be best for rooting (Jayanand et al. 2003).

The \textit{Agobacterium} mediated genetic transformation of \textit{Vigna mungo} has so far been reported with \textit{Gus} as a reporter gene and a selectable marker gene using the binary vector pCAMBIA 2301 and documented the presence of these genes by histochemical assay, PCR and Southern analysis (Karthikeyan et al. 1996; Saini et al. 2002; Saini and Jaiwal 2005; Saini and Jaiwal 2007; Acharjee et al. 2012; Sainger et al. 2015). Organogenesis from the embryonic axis triggering adventitious shoots after correct combination of hormone concentration, \textit{in vitro} selection with optimum concentration of antibiotic kanamycin monosulphate and rooting are crucial to genetic transformation. It is also difficult to recover the \textit{in vitro} raised shoots after several cycles of kanamycin selection, because most of the plants show hyperhydricity at the latter stages of subculture or do not survive during the process of hardening due to low frequency of rooting (Ghosh et al. 2017). The \textit{in vitro} regenerated shoots failed to produce roots on rooting media supplemented with high concentration of kanamycin during regeneration of putative transformants as reported by Krishna et al. (2010). In the present study the \textit{in vitro} selected shoots produced healthy and elongated roots in the optimized liquid half-strength MS media supplemented with 1.34 \( \text{mM} \) NAA. Karthikeyan et al. (1996) obtained resistance calli but could not regenerate plants from these calli. Saini et al. (2003) reported segregation of \textit{uidA} and \textit{nptII} gene into the \( T_1 \) progeny of the black gram plants in a Mandelian pattern of inheritance with a transformation frequency of 1% using cotyledonary node explants. The transformation frequency was further increased to 6.5% by using shoot apices excised from the pre-treated embryonic axes (Saini and Jaiwal, 2007). Genetic engineering employing ICPs of Bt (\textit{Bacillus thuringiensis}) provide resistance against Lepidopteran insects in crop plants (ISAAA, 2016). Various Bt genes (\textit{Cry1Ac}, \textit{Cry2Aa}, \textit{Cry1AcF}) were used for conferring resistance against pod borers in legumes like chickpea (Acharjee et al. 2010) and pigeon pea (Ghosh et al. 2017; Ramu et al. 2012). Till date, no agronomically important genes have been introduced into \textit{Vigna mungo} to transform it genetically. This is the first report of using a Bt gene for engineering black gram to confer insect resistance. Out of 9 plants, 4 transgenic plants were fertile, and produced a decent number of seeds. The \( T_1 \) progeny of three lines were grown in the greenhouse for further studies on transgene transmission and segregation. The PCR analysis of the \( T_1 \) progeny of all three lines (VM1, VM2 and VM3) confirmed transmission of the
transgene (Cry2Aa) in to segregating generation. The presence of 66KDa band on the western blot signifies the expression of Cry2Aa protein in the transgenic line VM3. A non specific band above 66KDa was also observed. Higher molecular weight bands are often seen when the target protein is posttranslationally modified. Acetylation, methylation, myristoylation, phosphorylation, glycosylation and ubiquitination are all modifications that increase the molecular weight of a protein.

In the present study, we have demonstrated an efficient *in vitro* regeneration protocol suitable to generate stable transgenic lines of black gram with a transformation frequency of 0.42% using *Agrobacterium* strain (Agl1) harbouring the Cry2Aa gene and a selectable marker gene, nptII. Using this protocol, attempt has now been made to generate large number of transgenic black gram harhourbing Cry gene so that one best stable transgenic line can be selected with high expression of transgene conferring complete protection against pod borers for its incorporation in the introgression breeding programme.

**Authors’ contribution**

Conceptualization of research (BKS, SA); Designing of the experiments (TK, BKS, SA); Contribution of experimental materials (BKS); Execution of field/lab experiments and data collection (TK, BB); Analysis of data and interpretation (TK, BKS, SA); Preparation of manuscript (TK, BKS, SA, AKH).

**Declaration**

The authors declare no conflict of interest.

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