# Retraction Notice

Title of retracted article: Agrobacterium Mediated Transformation of Vigna mungo (L.) Hepper with Cry1Ac Gene for Insect Resistance


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The paper does not meet the standards of "American Journal of Plant Sciences".

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# *Agrobacterium* Mediated Transformation of *Vigna mungo* (L.) Hepper with Cry1Ac Gene for Insect Resistance

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## Abstract

**Transfer of foreign genes into higher plants mediated by *Agrobacterium tumefaciens* is a standard technique in plant genetic engineering. We have produced of morphologically normal and fertile transgenic plants from leaf explants inoculated with *Agrobacterium tumefaciens* strain LBA 4404 carrying binary vector pCAMBIA 1319Z, the latter of which contains Cry1Ac gene for making insect tolerant blackgram plant, a neomycin phosphotransferase (nptII) gene and a *β*-glucuronidase (GUS) gene (uidA) for histochemical assay of the transgene. The transformed green shoots, selected and rooted on medium containing kanamycin, tested positive for nptII and uidA genes by histochemical assay and PCR analysis. These plantlets were established in soil and grown to maturity to collect the seeds. Analysis of $T_0$ plants showed the expression and integration of uidA into the plant genome. GUS activity in leaves, roots, flowers, anthers and pollen grains was detected by histochemical assay. For insect bioassay, the transgenic plants were toxic to bollworm larvae and lessened the damage caused by their feeding.**

## Keywords

**Blackgram (*Vigna mungo* L.) Genetic Transformation, *Agrobacterium tumefaciens*, Bt (Cry1Ac) Gene**

## 1. Introduction

Black gram [*Vigna mungo* (L.) Hepper] is an important leguminous source of protein for a large segment of the vegetarian population in the developing countries of Asia. The seeds of black gram contain 78% - 80% nitrogen

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in the form of albumin and globulin [1] [2]. In addition, being an important source of human food and animal feed, it also plays an important role in sustaining soil fertility by improving soil physical properties and fixing atmospheric nitrogen in symbiosis with soil bacteria of Rhizobium spp. India is the largest producer and consumer of black gram in the world, but production is limited due to its high susceptibility to lepidopteron pest [3]. At the pre harvest leaves are partially or completely chewed up by *Helicoverpa armigera* which is a polyphagous lepidopteron pest of worldwide Occurrence. Flowers and developing pods are eaten by *Heliothis virescence* & *Maruca* caterpillar which cause a large reduction in both yield and quality. These borer insets-pests can be controlled to some extent by the spray of different chemicals and pesticides before the harvest of black gram fruits but these chemicals have harmful effect on human health. Chemicals in the insecticides cause ecological imbalance and also accumulate toxic residues in the produces. In spite of all these realities, very limited effort has been made towards development of varieties resistant/tolerant to the attack of these lepidopteron in black grams. Genetic transformation of large-seeded legumes in general and *V. mungo* in particular has been difficult and challenging [4]-[10], although considerable progress has been achieved in some grains and legumes as reported in Pea by [11], in mungbean by [12] and in chickpea by [13]-[15].

Therefore the present study was planned with the objective to develop transgenic black gram plants resistant or tolerant to leaves, fruit and seed borer insect. The work was accomplished by expressing an insect-resistant *Bt* gene (*Cry*1Ac) under CaMV 35S constitutive promoter. It has been reported that *Bt* is not harmful to humans, other mammals, birds, fish, or even beneficial insects [16]-[18].

We report here, for the first time, the conditions for establishing an *A. tumefaciens*-based transformation protocol for the successful production of transgenic *V. Mungo* plants. This has been achieved through the development of an efficient method of plant regeneration through direct multiple shoot organogenesis from leave explants without cotyledons and the establishment of an optimal selection system.

## 2. Materials and Methods

Seeds of fourteen genotypes of black gram (*Vigna mungo* L.) namely, T-9, PS-1, Pusa-1, Pusa-2, Shekher-1, Shekher-2, Shekhar 3, Zetra-1, Zetra-2, Zetra-3, Zetra-4, Zetra-5, Zetra-6 and Zetra-7 were obtained from different localities with different characters such as T9 was Oval, & black, Pusa 1 & 2 were oval with greenish, Shekher 1 & 2 were oval and large with blackish green and Zetra 1, 2, 4, 5, 6 & 7 were oval and brownish black of Rajendra Agricultural University, Pusa which were sterilized for the *in vitro* experiments. The disarmed *Agrobacterium tumefaciens* strain LBA 4404 harbouring binary vector pCAMBIA1319Z, which contains a Cry1Ac gene, *β*-glucuronidase (GUS) gene (uidA) and a neomycin phosphotransferase gene (nptII) (**Figure 1**).

### 2.1. Explant Preparation

Seeds of *Vigna mungo* L. were washed under continuous flushing of running tap water for 30 min and then treated with a solution of the tween 20 (5% v/v) for 10 min and finally surface sterilized with $HgCl_2$ (0.1% w/v) for 10 min. Lastly, the seeds were rinsed thrice with autoclaved distilled water to remove any trace of contaminants. Seeds were germinated in MS medium [19] supplemented with 6-benzyl amino purine (BAP) at 2 $m \cdot g^{-1}$ (8.5 µM) ($MSB_2$).

### 2.2. Callus Induction and Maintenance

Primary leaves were excised from 7 days old seedlings, cut into 0.3 - 0.5 $cm^2$ segments and cultured on 30 ml MS medium with 3% sucrose, 0.8% agar, and different concentrations of 6-benzylaminopurine for callus induc-

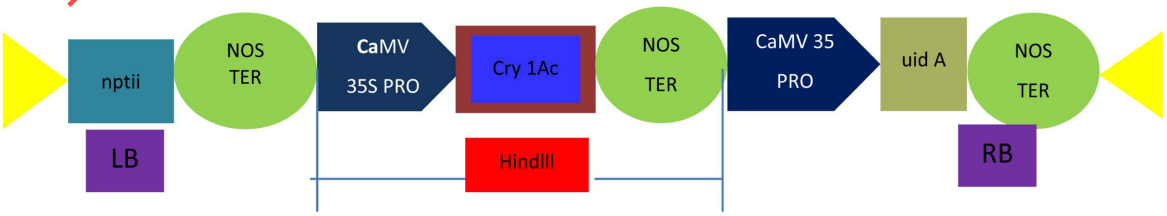



**Figure 1.** Vector construct map of Pk2Ac containing Cry1Ac gene.

tion. The callusing started after 12 days of inoculation and the pattern of the growth of callus was observed by measuring the diameter and growth percentage of the randomly selected callus at every 7 days intervals.

## 2.3. Culture Media and Conditions

All the media used in the investigation were supplemented with 3% sucrose and adjusted to pH 5.8 before autoclaving at 1.4 $kg \cdot cm^{-2}$ (121˚C) for 15 min**.** To prepare semisolid media, agar (Sigma, St. Louis, MO) at 8 $g \cdot l^{-1}$ was added before autoclaving. All the cultures were maintained under continuous white light (fluorescence density of 60 µmol $m^{-2} \cdot s^{-1}$) at 25˚C ± 2˚C.

## 2.4. Transformation and Plant Regeneration

*A. tumefaciens* strain LBA 4404 (pCAMBIA 1319Z) was grown on YEM (Yeast extract Mannitol-1.0 $g \cdot l^{-1}$ yeast extract, 10 $g \cdot l^{-1}$ mannitol, 0.1 $g \cdot l^{-1}$ NaCl, 0.2 $g \cdot l^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.5 $g \cdot l^{-1}$ $K_2HPO_4$, 15 $g \cdot l^{-1}$ agar) semi-solid medium containing 50 $mg \cdot l^{-1}$ kanamycin and 50 $mg \cdot l^{-1}$ rifampicin. A single bacterial colony was inoculated into 2 ml of liquid YEM containing the same antibiotics and grown overnight at 28˚C on a shaker at 200 rpm. A 200-µl aliquot of bacterial suspension was added to 20 ml of YEM liquid medium containing 50 $mg \cdot l^{-1}$ kanamycin and 10 $mg \cdot l^{-1}$ rifampicin and grown overnight. Bacteria were pelleted at 4000 rpm for 10 min and re-suspended in liquid medium containing MS salts, B5 vitamins [20], 3% sucrose and 0.5 µM BA, ($OD_{600}$ = 0.8). Primary leaves were excised from 7 days old seedlings, cut into 0.3 - 0.5 $cm^2$ segments and were gently stabbed before being immersed in bacterial suspension for 25 min with occasional shaking. Leaf discs were infected with *Agrobacterium tumefaciens* strain LBA 4404 (harbouring pCAMBIA binary vector with Cry 1Ac gene), co-cultivated and selected on fresh $MSB_2$ medium supplemented with Kanamycin 50 $mg \cdot l^{-1}$ and Cefotaxime 400 $mg \cdot l^{-1}$ and it formed callus in 4 weeks which was organized and subsequently formed a number of shoot buds. The putative transformed plants were established in soil and grown to maturity to collect $T_0$ seeds.

## 2.5. DNA Extraction

Total genomic DNA was extracted from fresh leaves of putative transformants ($T_0$) and non-transformed (control) plants by the Cetyltrimethyl ammonium bromide (CTAB) method [21].

## 2.6. Polymerase Chain Reaction Analysis

Putative transformants were screened by the polymerase chain reaction (PCR) for the presence of the Cry 1Ac gene. The 565-bp coding region of Cry 1Ac was amplified using 20-bp oligonucleotide primers (5'-ACAGAAGACCCTTCAATATC-3' and GTTACCGAGTGAAGATGTAA-3') (obtained from Merck acquired Bangalore Genei, India)

The amplification reaction was carried out using a thermal cycler (Biometra) under the following conditions: one cycle of 94˚C for 2 min; 30 cycles of 94˚C for 1 min (denaturation), 58˚C for 1 min (annealing), 72˚C for 2 min (extension); a final extension at 72˚C for 10 min (one cycle). The PCR was performed using approximately 100 ng of purified genomic DNA and Taq polymerase (Gibco-BRL, Gaithersburg, Md.). To ensure that reagents were not contaminated, DNA from non-transformed (control) plants was included in the experiments. The amplified products were separated by electrophoresis on a 1% agarose gel and visualized with ethidium bromide [22].

## 2.7. Histo-Chemical GUS Expression Assay

5-bromo 4-chloro-3-indolyl. *β*-glucuronide (X-gluc) was used as a substrate for histo-chemical localization of GUS activity [23]. 10 mg of X-gluc was dissolved in 1 ml of dimethylformamide and subsequently it was diluted in 50 mm phosphate buffer pH 7.0 to give final concentration of 1.0 mg/ml. The experiment tissues were incubated in freshly prepared X-gluc solution and incubated overnight at 37˚C in dark.

## 2.8. Analysis of Transgene Inheritance

The leaves of offspring of self-pollinated transformants were analysed for the presence of the Cry1Ac gene using PCR.

## 3. Results

Young, in vitro grown leaves on $MSB_2$ medium were transformed by infecting incised leaf surface with the *A. tumefaciens* strain LBA4404 containing the binary vector pCAMBIA1301-*Cry1Ac* (**Figure 1**). The callus (**Figure 2(a)** and **Figure 2(b)**), which was developed from leaves upon transfer to fresh $MSB_2$ semi-solid culture, formed nodular structure which later on developed into a number of shoot buds on 50 $mg \cdot l^{-1}$ kanamycin after 4 - 6 weeks under standard cultural conditions *i.e.* light intensity 60 $\mu \cdot mole \cdot m^{-2} \cdot s^{-1}$, photoperiod 16 h light and 8h dark and temperature 25˚C ± 2˚C (**Figure 2(c)** and **Figure 2(d)**). These shoot buds were separated from callus and transferred into MS basal medium supplemented with 0.1$mgl^{-1}$ NAA ad developed roots in 4 weeks from basal part of shoots (**Figure 2(e)**). These roots elongated (10 - 15 cm) and became sturdy and branched (**Figure 2(e)**) in 4 weeks and developed plantlets. The overall frequency of plantlet regeneration (**Figure 2(f)** and **Figure 2(g)** through the steps of callus formation, shoot buds development on the same medium and root development in different medium was 20.9% (18 plantlets/86 shoot buds). Under these conditions we were able to block necrogenesis following *Agrobacterium* co-cultivation. The stable transformed shoot buds were selected by periodic increase in kanamycin concentration. Initially after 20 days on medium containing 50 $mg \cdot l^{-1}$ kanamycin, a high number of shoot buds were obtained which later developed roots and develop plantlets. When transferred to medium containing higher concentrations of the antibiotic (75, 100 $mg \cdot l^{-1}$), many of the regenerants became yellowish, and were discarded after 50 days.

Of the developing kanamycin resistant transgenic plants, about 20% showed normal shoot and root development compared to a conversion frequency of 67% in the control plantlets. Nine regenerated transgenic lines (B-Cry-1, 2, 4, 5, 9, 10, 14, 15, 16, 17 and 18) were used for further analyses. Each transgenic line was derived from a single shoot bud and grew on kanamycin-containing media.

The putative transgenic plants had no phenotypic abnormalities in comparison to the untransformed control plants. Browning was controlled by the use of anti-oxidants (ascorbic and citric acid which reduces the action of polyphenol oxidase in the medium) so that leaf explants would form proliferated calli under *in-vitro* conditions without showing necrosis.



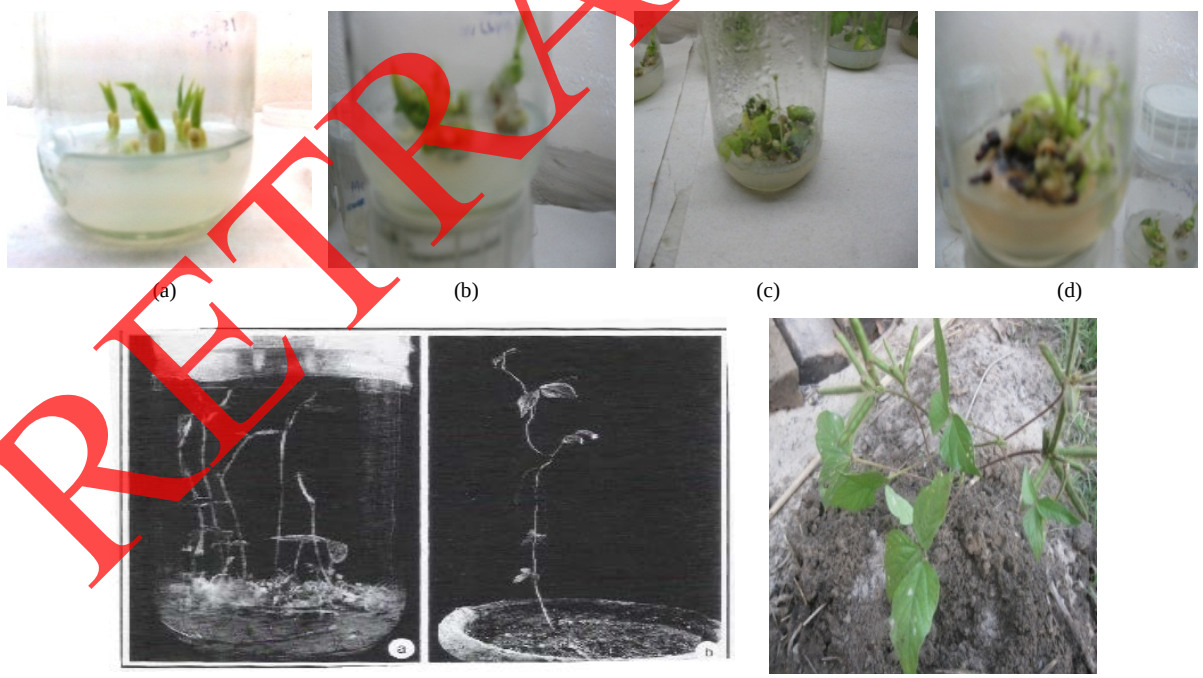



**Figure 2.** *Agrobacterium* mediated transformation in which transgene (Cry 1Ac) was integrated in its genome of putatively trnasformed blackgram plantlets-(a). Cotyledons with tender leaves; (b) from callus organised shoot buds; (c) shoot buds elongating; (d) mature shoot buds; (e) roots were organised from the base of shoot buds from rooting medium (f) acclimatized transgenic blackgram (g) fertile transgenic plants having many pods.

### 3.1. Integration of *Cry1Ac* Gene in Black Gram Plants

Southern blot analysis of transgenic plants clearly indicated the integration of *Cry1Ac* gene in plant genome. Gene integration was detected by gene specific probe after the plasmid pk2Ac DNA was digested with HindIII restriction enzyme. Plant genomic DNA digested with the same restriction enzyme and hybridized with *Cry1Ac* specific probe showed the integration of *Cry1Ac* gene in plant genome. Non-transformed T9 plant DNA was used as negative control while that of plasmid DNA pk2Ac was used as positive control.

### 3.2. Histochemical Assay of the Transgene (GUS) Expression

When explants were infected with *A. tumefaciens* with gene construct pCAMBIA having Cry1Ac & GUS, it was observed that whole explants turned blue colored in X-gluc substrate within 10 to 12 h. At this time, most of the GUS-positive colour appeared in undifferentiated tissue (**Figure 3**).

Polymerase chain reaction (PCR) of gene*Cry1Ac* confirmed the stable inheritance of these genes to subsequent generations. 565 bp internal fragments for *Cry1Ac* were amplified (**Figure 4**). No amplification was detected in negative control.

### 3.3. SDS-PAGE

Total protein was extracted from transgenic plants samples along with control and about 20 µg of extracted protein was loaded on 12% polyacrylamide gel. A 68 kDa band of *Cry1Ac* protein was observed in transgenic plant samples (**Figure 5**) and this specific protein band was absent in control sample.

### 3.4. Biotoxicity Assay and Field Infestation

Laboratory Biotoxicity assays with 2nd Instar *H. armigera* larvae showed that expression of both genes is sufficient to kill the targeted insects (**Figure 6**). In Laboratory Biotoxicity assay five lines namely, PS1, Pusa 1, Pusa

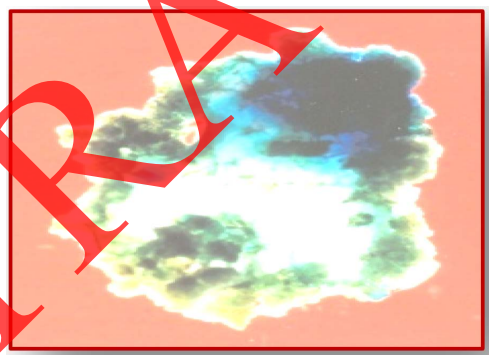


**Figure 3.** *Agrobacterium* mediated transformation of blackgram leaf tissue with gene construct having GUS gene shows blue colour in histochemical transient assay.

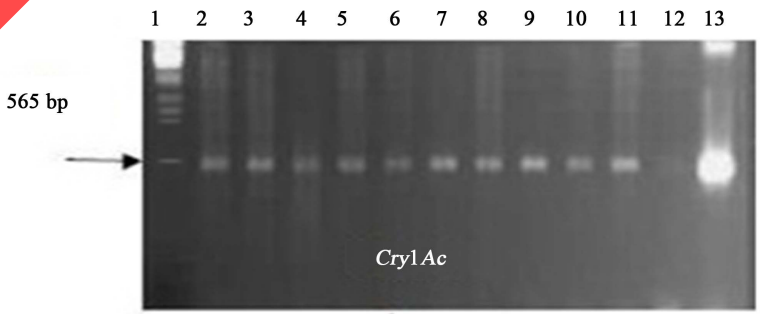



**Figure 4.** PCR amplification of *Cry1Ac*in transgenic plant. Lane 1: Lamda HindIII Marker; Lane 2 - 11: Representative transgenic plant of line PS1, Pusa1, Pusa 2, Shekher (Sh) 1, Sh 2, Sh 3, Zetra 1, Zetra 2, Zetra 3 and Zetra 4; Lane 12: −ve control (T9); Lane 13: +ve control (pk2Ac plasmid).

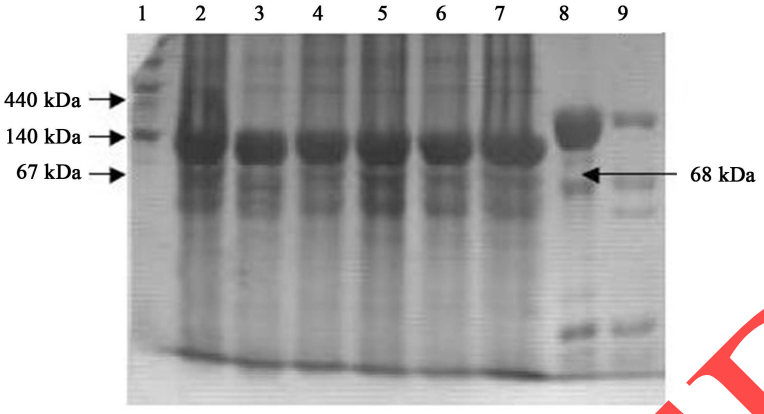



**Figure 5.** SDS-PAGE of extracted protein from different transgenic lines of black gram. Lane 1: High Molecular wt. Protein Marker, Lane 2 - 7: Transgenic plant of line PS1, Pusa 1, Pusa 2, Shekhar 1, Shekhar 2 , Shekhar 3, Lane 8: Positive Control (Isolated protein of *Bacillus thuringenesis* HD-73., Lane 9: Non Transformed Control, T9.

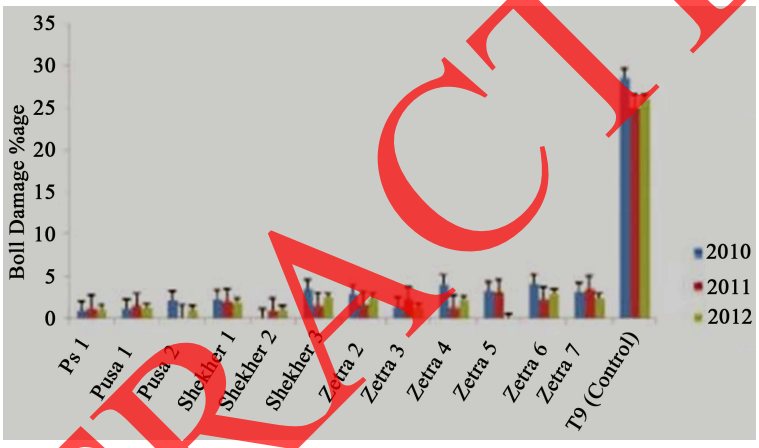
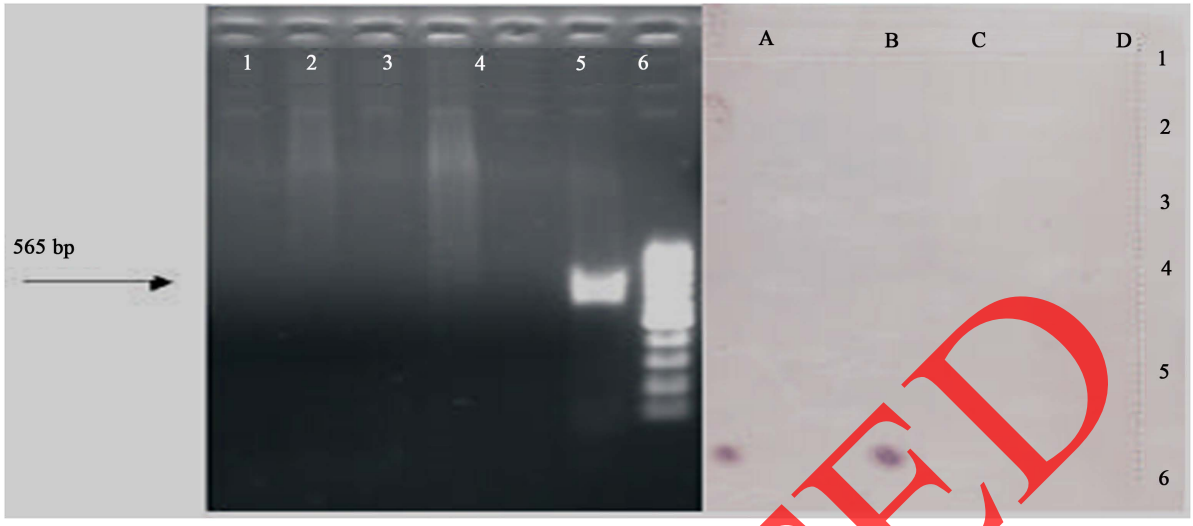



**Figure 6.** Graph showing boll damage % age in transgenic blackgram lines after infesting the crop artificially with 2nd Instar *Helicoverpa armigera* larvae.

2, Shekhar 1 and Shekhar 2 and were showing 100% mortality of larvae. While other lines showed a mortality rate of larvae that ranged between 70% - 90% as shown in **Figure 7(a)** & **Figure 7(b)**. The larvae which survived in few cases were too inactive or sluggish to be harmful for the plant. While in case of non-transformed control T9, no any mortality of larvae was noted. Field infestation was carried out twice *i.e.* in August and September respectively. These months were selected for the infestation as boll worm activity was maximum during these months in Bhagalpur, India. Appreciable level of resistance against the targeted insects and boll damage varying between 0.09% - 4.5% in case of transgenic lines was noted while in control T9, boll damage was calculated upto 25%. (**Figure 8**). Transgenic lines also varied in boll damage percentage when analyzed statistically transgenic Shekhar-1 showed excellent result regarding boll damage percentage. The resistance against lepidopterans was stably inherited during both years and all plants showed high level of resistance against lepidopterans.

## 4. Discussion

The expression of Cry1Ac gene through *Agrobacterium*-mediated transformation and tolerant to bollworm *(Helicoverpa armigera*) insect was first reported in cotton [24], Transgenic potato was formed by the entry of Cry 1la1(Cry V) gene into potato genome and become tolerant to potato tuber moth [*Phthorimaea operculella* and *Symmetris chematangolias* (Lepidoptera: Gelechiidae)] [25], transgenic brinjal was made by the expression of Cry 1Ab gene into brinjal through *Agrobacterium* mediated transformation and tolerant to fruit. Transgenic




(a) (b)

**Figure 7.** PCR and Dot blot assay to confirm the horizontal gene flow. Polymerase chain reaction (PCR) was performed using gene specific primers to detect the persistence of *Cry1Ac* gene in soil particles. There was no detection of Cry gene in soil samples (1 - 4 lanes) while positive sample plasmid pk2Ac amplified a required 565 bp Fragment (5 lane) and (6 DNA marker lane). No integration of *Cry* genes in dot blot assay (A-D lanes) did not detect cry protein in soil sample suggested that *Bt* Blackgram is environment friendly (**Figure 7**).

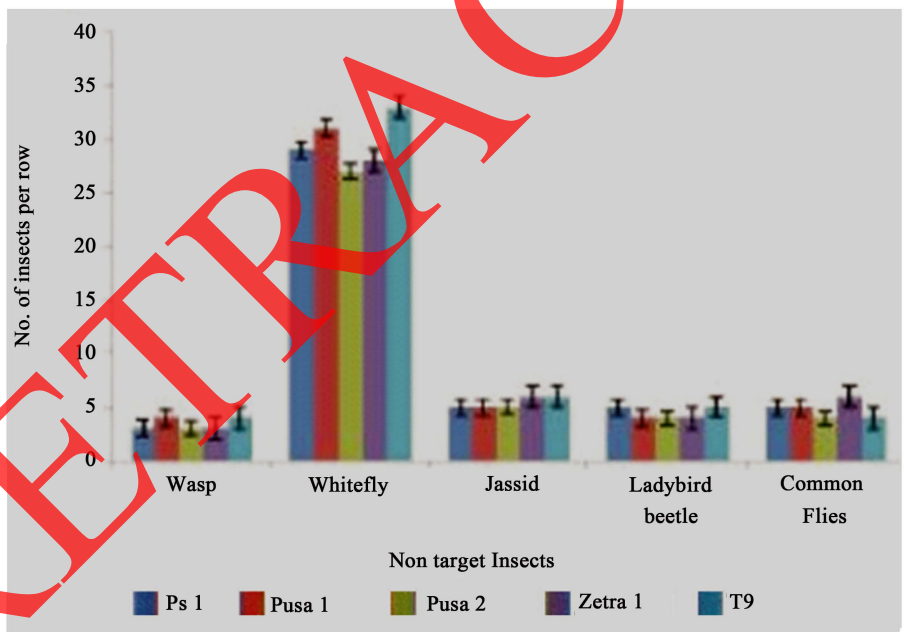



**Figure 8.** Effect of Bt black gram on non-target insects.

black gram lines encoding two insecticidal crystalline proteins from *Bacillus thuringiensis* can reduce the use of conventional broad-spectrum pesticides dramatically against targeted pests but there is always a risk that insects could become resistant to *Bt* toxin after prolonged and repeated field exposure. Therefore the in the present work the performance of advance blackgram lines against targeted pests was studied for three consecutive years (2010, 2011 and 2012) (**Table 1**) and was found that Cry1Ac gene could be the best way to delay the resistance level in insect pests against endotoxins. Performance of these lines with Cry1Ac gene has been excellent regarding the insect resistance and yield performance.

Some of the transgenic lines (PS1, Pusa 1, Pusa 2, Shekhar 1 and Shekhar 2) showed up to 100% resistance

**Table 1.** Comparison of yield contributing factors between transgenic lines and untransformed T9 (2010-2012).

| | Average number of bolls/plant | | | Boll damage %ge | | | Days of mature | | | Average yield/plant (g) |
|---|---|---|---|---|---|---|---|---|---|---|
| Line no. | 2010 | 2011 | 2012 | 2010 | 2011 | 2012 | 2010 | 2011 | 2012 | |
| PS 1 | 42.1 | 42.2 | 43.6 | 0.91 | 1.21 | 1 | 109 | 114 | 114 | 140.4 |
| PS 2 | 43.1 | 45.2 | 42.2 | 1.19 | 1.46 | 1.27 | 109 | 114 | 114 | 139.2 |
| Pusa I | 37.5 | 39.4 | 41 | 2.2 | 0.08 | 1.04 | 110 | 115 | 115 | 136.4 |
| Pusa II | 43.4 | 45.4 | 40.8 | 2.28 | 2.04 | 1.86 | 110 | 115 | 115 | 139.7 |
| Shekhar 1 | 53.1 | 56.7 | 55.4 | 0.09 | 0.95 | 1.05 | 110 | 115 | 115 | 152.1 |
| Shekhar 2 | 42 | 40.7 | 43.4 | 1.5 | 1.55 | 2.5 | 110 | 115 | 115 | 124.4 |
| Shekhar 3 | 41.8 | 41 | 38.3 | 2.82 | 1.65 | 2.43 | 110 | 115 | 115 | 124.3 |
| Zetra 1 | 36 | 38.1 | 37.4 | 1.38 | 2.24 | 1.33 | 110 | 115 | 115 | 111.2 |
| Zetra 2 | 35 | 40.1 | 37.5 | 4.04 | 1.28 | 2.15 | 125 | 125 | 125 | 112.4 |
| Zetra 3 | 39 | 38 | 36 | 3.3 | 3.12 | 2.4 | 110 | 115 | 115 | 115.4 |
| Zetra 4 | 36.8 | 42.5 | 38.8 | 4.12 | 2.25 | 3 | 125 | 125 | 125 | 118.5 |
| Zetra 5 | 34.5 | 37.5 | 34.4 | 3.17 | 3.52 | 2.42 | 125 | 125 | 125 | 118.5 |
| T9 (Control) | 26.9 | 27.2 | 25.4 | 28.5 | 25 | 26 | 150 | 150 | 150 | 94.2 |

Numbers with same letters within columns are not significantly different from each other according to LSD test at 5% level of significance.

against boll worm resulting in almost zero damage in the bolls and while few of them had 70% - 90% resistance against targeted pest. Similarly boll damage in few lines was almost zero showing 100% resistance to boll worms especially *Helicoverpa armigera*. Difference in resistance level in laboratory biotoxicity assay and boll damage percentage in field is perhaps due to the expression level of insecticidal protein which varies with the age of plant as well as in different plant parts.

Transgenic lines having Bt gene (Cry1Ac) were superior as compared to untransformed control variety with respect to average number of bolls, boll damage percentage, early maturing and yield per plant. Results suggested stable integration of these insecticidal genes in subsequent generation as revealed by different molecular analysis.

Transgenic lines expressing *Bt* (*Cry1Ac)* gene provided protection against lepidopteran insects throughout the growth period. These lines provided high resistance against targeted pests till the harvest of the crop and were desirable in agronomic and morphological characteristics. It is very important to note that these are advance lines in T1 to T2 generations, insecticidal genes have been inherited stably and no gene silencing has been observed for Cry genes.

## 4.1. Pcr and Dot Blot Assay

To confirm the expression of C*ry* gene, a dot blot assay was performed that did not detect *Cry* protein in soil samples suggesting the degradation of C*ry* protein after crop harvesting. Only Positive protein samples isolated from soil bacterium *Bacillus thuringiensis* strain (HD-73) were detected (**Figure 7**). No other detection was found in test samples as well as in negative samples.

## 4.2. Risk Assessment Studies

Almost same number of non-target insects was found both on transgenic and non-transgenic lines. The data was found non-significant when analysed statistically rejecting the hypothesis that transgenic crops can affect the survival of the non-target insects or natural enemies of insects (**Figure 8)**.

Most of the predators on-target insects like ladybird beetle and red cotton bug, we found that gene could be making the crop tolerant to the wasp and spiders were found throughout the season of crop growth. The transgenic blackgram plants were insect tolerant and high productivity in comparison to untransformed blackgram plants (**Table 2**).

**Table 2.** The MSP (Market support price) of blackgram for the last 3 years.

| | Mean SD unit: Rs/Kg | Mean SD unit: Rs/Kg | Mean SD unit: Rs/Kg |
|---|---|---|---|
| Pulse | 2010 | 2011 | 2012 |
| Urad (untransformed T9) | 43.00 ± 0.47 | 43.33 ± 0.15 | 46.20 ± 0.1 |
| Urad (transformed Pusa, Shekhar, Zetra) | 48.01 ± 0.01 | 48.76 ± 0.23 | 48.90 ± 0.53 |

SD: Standard deviation.

## 5. Conclusion

From the above documents we come to the conclusion that the transgenic blackgram in which transgene (Cry1Ac) has been inserted into blackgram genome via *Agrobacterium* mediated transformation, shows tolerant to insects and high productivity. From molecular and T0, T1 and T2 generations analyses confirmed that crystal protein gene is stable integrated into their genome. It is also tested that crystal protein is highly toxic to lepidopteron and not harmful to human being [26] and through PCR and dot blot assay also confirms that crystal protein is not coming to soil to contaminate it so that this method is good for transgenesis and ecofriendly.



## References

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