# Standardization of *In-vitro* Genetic Transformation Technique in Chickpea (*Cicer arietinum* L.) for Pod-borer Resistance

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**Abstract**

**Here we report the establishment of an efficient Agrobacterium- mediated genetic transformation in chickpea (*Cicer arietinum* L.) using *cryIIAa* gene of *Bacillus thuringiensis*. Precultured single cotyledonary node along with embryo axes (EA1C) were injected by Agrobacterium suspension of OD 600 = 0.3-0.5, co-cultivated for 48 h on precultured medium containing 0.5 mg $l^{-1}$ TDZ and 400 mg $l^{-1}$ Cefotaxime without selection pressure. For the selection of transformants 1000 mg $l^{-1}$ kanamycin was employed on well established plants by using different explants from putatively transgenic plants. The percentage of the kanamycin resistant plants varied from 6.62 to 16.12%. The results of PCR, RT-PCR and further Southern blotting of genomic DNA of kanamycin resistant plants showed that the synthetic gene *cryIIAa* had been integrated into the genome of some transformed plants. The transgenic plants with *cryIIAa* gene of *B. thuringiensis* showed improved tolerance than the controls by insect bioassay. *cryIIAa* gene inherited in some transgenic lines as Mendelian segregation pattern. The results of the present study indicate that highest transformation frequency (15.05%) could be achieved by Agrobacterium-mediated transformation.**

**Keywords:** *Cicer arietinum*, *cryIIAa*, Agrobacterium-mediated genetic transformation, single cotyledon with mature embryo axes.

## Introduction

Gene transfer into plants has opened new ways for theuse of recombinant DNA technology in physiological and molecular studies and may be useful in complement ing conventional breeding programme. Advancement in molecular genetics in grain legumes for gene expression, T-DNA tagging, promoter analysis for crop improvement requires efficient genetic transformation systems. However, grain legumes have been considered to be recalcitrant for genetic transformation, since plant regeneration, which is a prerequisite for transgenic plant development is a major limiting factor (Somers *et al*. 2003). Chickpea (*Cicer arietinum* L.) is an important grain legume of the 40 countries and is major source of proteins for the population of developing countries, however, shows high incidence of pest and fungal attack (Sonia *et al*. 2003, Romeis *et al*. 2004). Among these, gram pod borer (*Heliothis armigera*) has been reported to cause losses ranging from 20-80 % in different parts of country (Singh *et al*. 2006). Conventional plant breeding approaches, due to limitations in availability of gene pool, could not provide new varieties, which are resistant to *Heliothis armigera*. The unscientific control measures adapted against these pests by farmer is to inefficient to control this pest. This has also lead to development of pesticide resistance in insects (Kumar *et al*. 1995, Indurkar *et al*. 2007, Sharma and Ortiz 2000).

*Agrobacterium*-mediated transformation has been used successfully in grain legumes for over a decade (Christou 1995). Efficient transformation system for pea was developed based on direct shoot regeneration and meristem proliferation from Agrobacterium treated seedling explants (Schroeder *et al*. 1993). Like other leguminous crops, meristematic cells of apical meristem and cotyledonary node of embryonic axes of chickpea has been used for genetic transformation by earlier workers (Kar *et a*l. 1997, Kar *et al*. 1996, Krishnamurthy *et al*. 2000, Polowick *et al*. 2004, Senthil *et al*. 2004, Sarmah 2004, Tewari-Singh *et al*. 2004 and Sanyal *et al*. 2005). For the

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development of insect-resistant plants, although a number of insect-resistant genes are reported to encode for toxins that target lepidopteran larvae, *Bt* toxin received the maximum attention. *Heliothis armigera*, the range of *Bt* toxin with *CryIIAa* and also effective against dipteran insects. Hence, it is desirable to introduce *cryIIAa* gene into chickpea to impart insect resistance.

**Methodology**

Seeds of chickpea (*Cicer arietinum* L) cv. Annigeri 1 were provided by the Department of Genetics and Plant Breeding, University of Agricultural Sciences, Dharwad (Karnataka), India. The seeds were surface sterilized in 70% ethanol followed by 0.1% mercuric chloride (w/v) for five minutes. The sterilized seeds were rinsed for five times with sterile distilled water and left to soak in sterile distilled water for 12 h. The testas were removed and the single cotyledon along with mature embryo axes germinated for 48 h under 16/8 h (light/dark) photoperiod on basal media containing MS (Murashige and Skoog, 1962) salts, 0.8% agar and supplemented with 0.5 mg $l^{-1}$ Thidiazuron (TDZ).

The disarmed and hyper virulent *Agrobacterium tumefaciens* strain EHA 105 harboring P*BinBt3* was used as a vector system for in-vitro transformation. Plasmid P*BinBt3* contains the *cryIIAa* gene linked the cauliflower mosaic virus (CaMV) 35S promoter and neomycin phosphotransferase (*npt II*) gene under the control of napolene syntheses (nos) promoter and terminator *npt II* was used as a selectable marker.

Four different methods of colonization and co-cultivation were followed.

MT1- EA1C (single cotyledonary node along with embryo axes) were injured by *Agrobacterium* and co-cultivated for 48 h.

MT2- EA1C were injured mildly and dipped in *Agrobacterium* for 20 min and co-cultivated for 48 h.

MT3- EA1C were dipped for 30 min in *Agrobacterium* and co- cultivated for 3 days.

MT4- EA1C precultured in the medium supplemented with 0.5 mg $l^{-1}$ TDZ for 48 h, bacterial suspension culture was injected and co-cultivated for 48 h on same media. Co-cultivated EA1C explants were rinsed in liquid basal MS medium fortified with 400 mg $l^{-1}$ cefotaxime. Then the explants were blotted on sterile blotting paper to remove excess bacteria and moisture and transformed to shoot induction medium (MS + 0.5 mg $l^{-1}$ TDZ + 400 mg $l^{-1}$ cefotaxime) without selection pressure. As we found difficulty in elongating the induced multiple shoots on shoot induction medium, low concentration of cytokinin like MS supplemented with 0.5 mg $l^{-1}$ BAP was used. Elongated individual shoots were separated and transferred to root induction media like ½ MS supplemented with 0.2 mg $l^{-1}$ IBA, 200 mg $l^{-1}$ cefotaxime and 2% sucrose. All explants were maintained in the culture room at 22°C under 16/8 h (light/dark) photoperiod. Rooted shoots were transferred to cups containing sterilized vermiculite and kept in growth chamber by covering with polythene bag for a week and then transferred into hardening medium containing 1:1:1 soil, sand and vermiculite. Antibiotic sensitivity test were conducted by excising explants like shoot tip and leaf blade from well established plants and subjected into the MS supplemented with 1000 mg $l^{-1}$ kanamycin (Himedia).

The integration of *cryIIAa* gene was ascertained by PCR amplification using gene specific primers, RT-PCR and Southern Blot hybridization. Genomic DNA was isolated form all well established kanamycin resistant plants by using the CTAB method (Edwards *et al*. 1991). DNA pellet was dissolved in TE buffer and the concentration of DNA was monitored spectrophotometriclly. PCR amplification was carried out with genomic DNA from putative transgenic plants, control plants and plasmid DNA as templates for amplification of *cryIIAa* gene. Primers used were- 5' GGGCACTGTGTCCTCCTTCCTCCTC 3' (F) 5' GGGGAGATGGTGAAGCCGGTC TAG 3' (R) which amplified a 1.2 kb fragments.

Transgenic expression analysis of PCR positive plants were done by two step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The total RNA from transgenic and control plants were isolated using Eppendorf RNA isolation kit (Eppendorf Hamburg Germany) and two step RT-PCR using cMaster Tm RT plus system and cMaster TM RT kit (Eppendorf Hamburg Germa-

ny). Equal amount of total RNA from both transgenic and control chickpea plants were used for RT-PCR reaction and reaction mixture were prepared as per manufactures instructions. RT-PCR product was fractioned by 1% agarose gel electrophoresis and visualized in gel documentation system (BiovGel 1D). For Southern hybridization analysis for X ba I restricted genomic DNA was used to detect number of copies of gene integration Radiolabelled *cryIIAa* gene probe.

Insect mortality bioassay was carried out using protocol as described earlier (Ratnakar et al, 1994). In brief second instar larvae of *Heliothis armigera* were used in the experiment. Larvae were starved for 24 h before feeding on leaves of transgenic and non transgenic plants. Feeding was allowed for 3 days and data were taken on larval weight, survival ability and mortality.

**Results and Discussion**

A major application of gene transfer technology is the introduction of agronomical useful traits into crop plants. Even though there are major reports available on the introduction of *cryIA(c)* gene (Kar *et al*. 1997) into chickpea to protect crop from lepidopteron insects in field conditions. Reports of resistance to lepidoptera and diptera together are scanty (Sarmah *et al.* 2004). Transgenic plants could be obtained from any explants following their cocultivation with *Agrobacterium* but large number of multiple shoots proliferates from axillary buds and it ensures maximum genetic uniformity (Horsch *et al*. 1985). In the present experiments, when three explants such as mature embryo axes (MEA), shoot apical meristem (SEM) and embryo axes with single cotyledon (EA1C) were used, EA1C produced the highest response as well as more number of multiple shoots. Transformation of gene through cotyledonary node has also been reported in Soybean (Donaldson and Simmonds 2000), Peageon pea (Lawrence *et al*. 2001), Pea (Bean *et al*. 1997), Groundnut (Chang *et al*. 1994) and Vigna species (Jaiwal *et al*. 2001). Selection of a highly regenerable explant such as EA1C from cv. Annigeri 1 and by optimizing the different parameters for Agrobacterium injection has resulted in high frequency of transformation in to the present study. When we used mature embryo axes and shoot apical meristem regeneration, the frequency of transformation was low. For most of the experiments on transformation, mature embryo axes with single cotyledon was transferred to the shoot regeneration medium (MS + 0.5 mg $l^{-1}$ TDZ + 200 mg $l^{-1}$ cefotaxime) shoots increased number of multiple shoots (41). The control explants which were subjects to only MS medium or along with other growth regulators (Zeatin and BAP) did not show more response. TDZ has been previously used for shoot induction in several plant species like dry bean (Malik and Saxena 1992) and other crops (Kambale *et al*. 2003 and Jayanand *et al*. 2003). The putatively transformed shoot buds when subcultured on basal MS medium supplemented with lower level of cytokinin (0.5 mg $l^{-1}$ BAP) showed highest response (75.5%) for elongation of bunch of shoots and more than 5 shoots were elongated. It indicates that use of primary and secondary medium for shooting has been essential in chickpea (Senthil *et al*. 2004). About 90% of regenerated shoots showed well-developed roots in ½ MS supplemented with 0.2 mg $l^{-1}$ IBA, 200 mg $l^{-1}$ cefotaxime and 2% sucrose. The complete plants were hardened in the growth chamber containing soil, vermiculite and sand in equal proportion for 2 weeks and transferred to the soil. The two main deficiencies of *in-vitro* grown plants are poor control of water loss and hydrotropic mode of nutrition was observed. Hence gradual acclimatization is necessary to these plants to survive transition from culture to the green house.

Different concentration of kanamycin (0, 50, 100, 200, 400, 800, 1000 and 1200 mg $l^{-1}$ ) were tested on non transformed leaf blades derived from well established single cotyledon alongwith embryo axes to determine the lethal concentration for the selection of transformed shoots. Kanamycin at 800 mg $l^{-1}$ had little effect on shoot growth, while complete albino was observed at 1000 mg $l^{-1}$ Kanamycin (Table 1) (Figure 1). The leaf blades excised from well established putatively transgenic plants were subjected into basal MS medium supplemented with 1000 mg $l^{-1}$ kanamycin showed 16.12 % resistance in MT4 method. While MT1, MT2 and MT3 showed 6.80%, 6.62% and 0% respectively (Table 2). These levels are significantly higher than those obtained

**Table 1**. Sensitivity to kanamycin

| Concentration of kanamycin (mg $L^{-1}$) | Mature embryo axis (MEA) | Shoot tip (SAM) | Leaf blade |
|---|---|---|---|
| MS+0 | ++ | ++ | ++ |
| MS+50 | ++ | ++ | ++ |
| MS+100 | ++ | ++ | ++ |
| MS+200 | ++ | ++ | ++ |
| MS+400 | + | ++ | ++ |
| MS+600 | + | + | + |
| MS+800 | + | + | + |
| MS+1000 | 0 | 0 | 0 |
| MS+1200 | 0 | 0 | 0 |

++: Normal growth; + : Stunted growth; 0 : Albino

**Table 2**. Frequency of putative transgenic plants.

| Method | No. of plants established | Survival (%) | No. of Kanamycin resistant plants | PCR positive plants | Transformation frequency (%) |
|---|---|---|---|---|---|
| MT1 | 147 | 26.72 | 10 (6.80%) | 4 | 2.72 |
| MT2 | 181 | 30.18 | 12 (6.62%) | 6 | 3.31 |
| MT3 | 18 | 3.00 | 0 (0.0%) | 0 | 0.00 |
| MT4 | 186 | 77.50 | 30 (16.12%) | 28 | 15.05 |

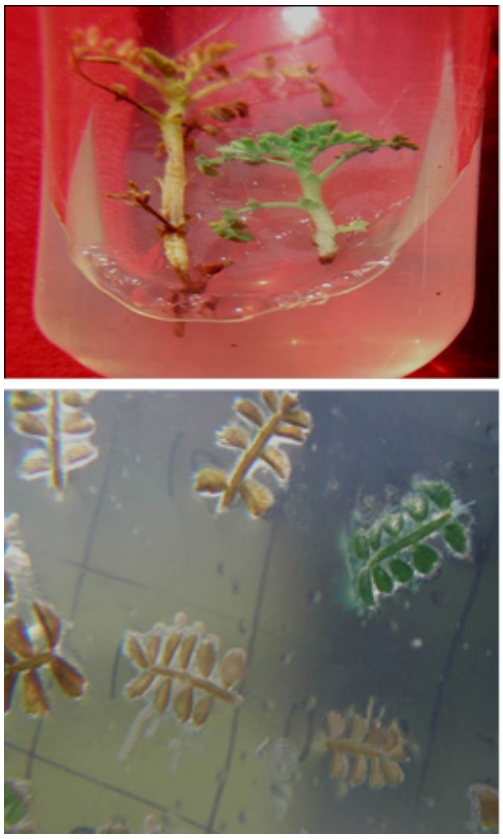

**Figure 1**. Sensitivity to kanamycin

from other seed derived explant types and selection schemes, which are described elsewhere (Chakrabarty *et al*. 2002). A total of 30 lines of putative transgenic as identified by selection on media were identified from experiments using the protocol described in this paper (MT4). These clones were morphologically normal and produced seeds. Several plants represented many clones. The process of transformation from inoculation with Agrobacterium to produce transgenic seed was short taking an average of 4-6 months with a minimum of 4 months to produce $T_1$ seeds.

PCR amplification results obtained from kanamycin resistant plants showed that all putatively transformed plants with P*BinBt3* plasmid produce amplified fragments of 1.2 kb, the expected size of PCR product with *CryIIAa* specific primers while control plants showed no amplification (Figure 2). Among the different methods, 2 days preculturing of EA1C followed by *Agrobacterium* injection in nodal region and 2 days co-cultivation accelerated transformation frequency to 15.05%. The frequency of transformation reported in other studies ranged from less than 0.4% (Krishnamurthy *et al*. 2000) to 13.3 % (Senthil *et al*. 2004, and Sanyal *et al*. 2005) in *Agrobacterium*-mediated transformation.

Expression analysis by RT-PCR of few plants selected from different events showed expression of *cryIIAa* gene. This implies expression of transgenic at transcribed level. Kanamycin resistant plants thus obtained in the present study were confirmed as true transforma nts.

Stable integration of these genes in the chromosome of kanamycin resistant plants obtained through independent transformation events was shown in the present study by Southern blot hybridization using *cryIIAa* gene probe (Figure 3). Southern hybridization results showed identical pattern of hybridization signals, thus confirming the integration of transgene in plant genomes, which could probably due to presence of a hot spot for integration in the plant genome of chickpea. Earlier studies have also presented results on Southern hybridization of genomic DNA digested with *Hind III* (Indurkar *et al*. 2007 and Kar *et al.* 1997), which showed that transgenic plants have identical signal on Southern hybridization.

Young leaves of transgenic plants were subjected to insect feeding assay. Plants expressing *CryIIAa* protein were tested by using leaves for feeding the assay with second instar larvae of *Heliothis armigera*. Data indicated that To chickpea plants expressing *CryIIAa* protein exhibits significant reduction in the weight of larvae and also showed high mortality compared to larvae fed on control plants (Table 3). Larvae fed on transgenic plant parts had stopped feeding and most of the plant parts remained unaffected, whereas the larvae on untransformed plants fed voraciously (Figure 4). These results are in accordance with the studies conducted earlier, when larvae showed different feeding patterns and growth in transgenic leaf diseases of cabbage and chickpea respectively (Indurkar 2007:Chakrabarty *et al*. 2002).

Transmission of transgenes into the progeny was investigated by following the inheritance of the *cryIIAa* gene in 11 $T_1$ progeny plants using PCR and RT-PCR (Table 4). The overall segregation ratio among progeny of plants from most of the lines appeared to be close to a 3:1 Mendelian ratio, indicating integration of transgene at single locus. The $\chi^2$ test indicated a significant difference from the expected 3:1 ratio in two lines suggest-

**Table 3**. Mortality and inhibition of growth of *H. armigera* larvae by feeding on $T_0$ and $T_1$ plants.

| Plant No. | Cumulative mortality (%) | | | Corrected mortality (%) | Per cent loss in body weight of larvae | | |
|---|---|---|---|---|---|---|---|
| | 24 h | 48 h | 72 h | | 24 h | 48 h | 72 h |
| 4 | 0 | 64.14 | 80 | 100 | 24.03 | 42.90 | 56.03 |
| 5 | 0 | 75.66 | 80 | 100 | 18.07 | 40.03 | 51.13 |
| 8 | 0 | 80.59 | 80 | 100 | 14.73 | 38.14 | 49.51 |
| 11 | 0 | 67.80 | 80 | 100 | 14.86 | 42.28 | 55.19 |
| 14 | 0 | 69.47 | 80 | 100 | 17.10 | 40.53 | 60.17 |
| 15 | 0 | 69.47 | 80 | 100 | 18.16 | 47.00 | 55.53 |
| 71 | 0 | 69.47 | 80 | 100 | 12.37 | 36.04 | 53.83 |
| 78 | 0 | 73.66 | 80 | 100 | 13.74 | 35.38 | 57.19 |
| 79 | 0 | 76.33 | 80 | 100 | 14.68 | 41.11 | 58.64 |
| 80 | 0 | 73.80 | 80 | 100 | 16.61 | 37.19 | 49.51 |
| Control | 0 | 0 | 0 | 20 | 22.24 | 78.14 | 193.07 |
| | | | | | | S.Em ± | CD (5%) |
| | | | | | Plants | 1.026 | 3.80 |
| | | | | | Period | 1.97 | 2.30 |
| | | | | | P x P | 0.59 | 2.23 |

**Table 4**. PCR and expression analysis of progenies of $T_1$ plants.

| Sl. No. | Pedigree of T1 plant | No. of seeds Tested | Positive | Negative | Ratio | $\chi^2$ Value |
|---|---|---|---|---|---|---|
| 1 | 140-1 | 28 | 20 | 8 | 3:1 | 0.39 |
| 2 | 142-1 | 21 | 16 | 5 | 3:1 | 0.10 |
| 3 | 146-1 | 27 | 20 | 7 | 3:1 | 0.018 |
| 4 | 115-1 | 10 | 7 | 3 | 3:1 | 0.016 |
| 5 | 98-1 | 32 | 25 | 7 | 3:1 | 0.033 |
| 6 | 175-1 | 34 | 23 | 11 | 3:1 | 1.64 |
| 7 | 153-1 | 18 | 13 | 5 | 3:1 | 0.44 |
| 8 | 108-1 | 33 | 20 | 13 | - | 5.47 |
| 9 | 186-1 | 19 | 14 | 5 | 3:1 | 0.013 |
| 10 | 117-1 | 17 | 13 | 4 | 3:1 | 0.49 |
| 11 | 205-1 | 22 | 12 | 10 | - | 4.92 |

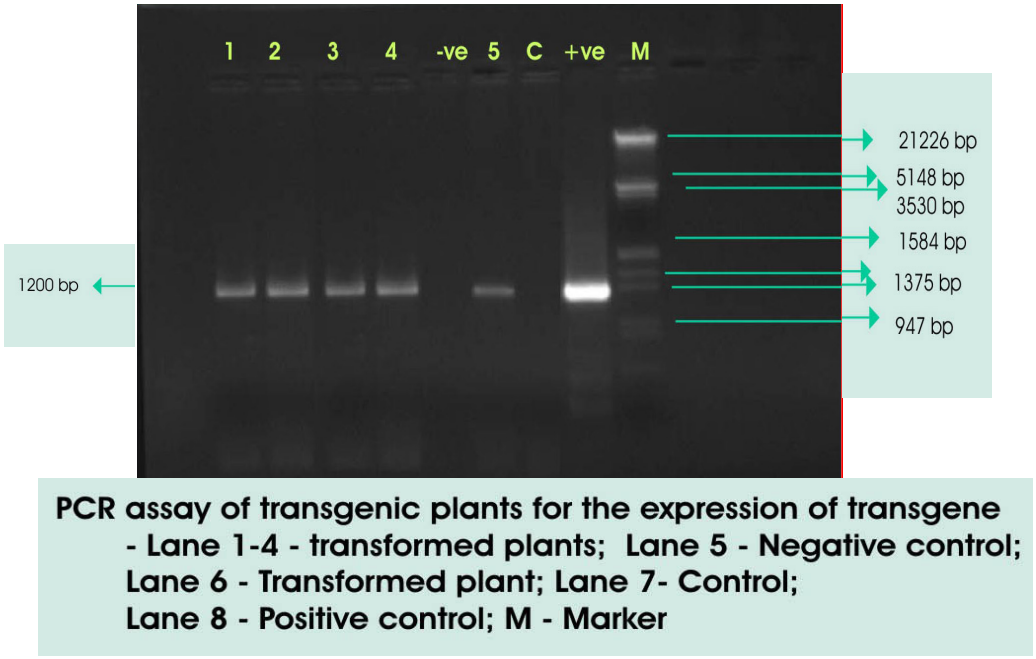


**Figure 2**. PCR assay of transgenic plants

ing either possible partial silencing the *cryIIAa* gene, a mutated insertion sequence , chimerism in the To line or an imperfect kanamycin resistant test (Stam *et al*. 2000 : Ma and Mitra 2002).

Advancement in molecular genetics of grain legumes, especially chickpea for gene integration, promoter analysis and expression of genes for crop improvement require efficient genetic transformation methods. Hence, development of simple, rapid, high frequency transformation system in chickpea by *Agrobacterium* as has been shown in the present study, will benefit further studies. Compared to low transformation frequency in earlier studies using *Agrobacterium*- mediated transformation of chickpea (Polowick *et al*. 2004, Sarmah 2004, Sanyal *et al*. 2005), the present study reports high fre-

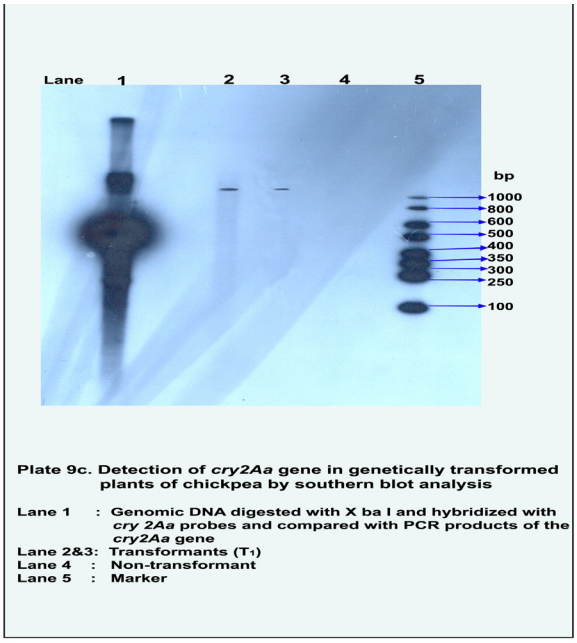


**Figure 3**. Detection of CryIIAa gene in genetically transformed plants of chickpea by southern blot analysis.

quency transformation in *Cicer arietinum* cv. Annigeri 1 by preculturing EA1C in MS + 0.5 mg $l^{-1}$ TDZ and injecting Agrobacterium and co-cultivated for 48 h in preculturing media without selection pressure at early stage.

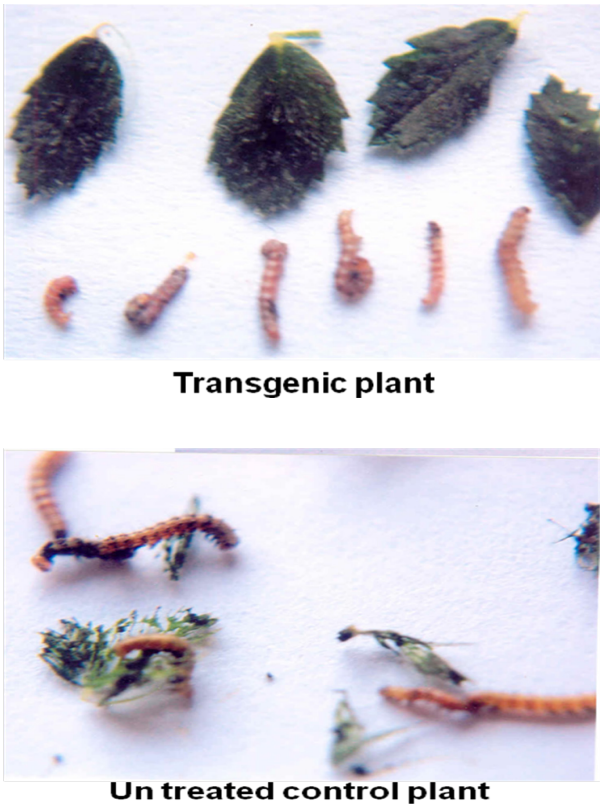


**Figure 4.** Insect bioassay of Bt plants

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