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A high throughput bioassay system for screening of Bt transgenic plants expressing Cry proteins

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Abstract
In the present study a rapid screening technique was developed to identify transgenic progenies that are resistant to a target pest at seedling stage under greenhouse conditions. Seedlings of 10 day-old insect resistant transgenic cotton and 30 day-old brinjal were used for bioassay. Two and four days after the release of neonate larvae in cotton and brinjal, respectively, seedling bioassay could distinguish susceptible plants from the resistant ones. The seedling bioassay procedure can give a rapid and early assessment of insect resistance, significantly reducing the time required for validation of genes of interest. This bioassay has potential as a rapid method for screening transgenic cotton and brinjal for insect resistance as well as evaluating the effects of insecticidal proteins on the larvae.

Keywords: Insect resistance, seedling bioassay, *Spodoptera litura, Helicoverpa armigera*, *Leucinodes orbonalis*

1. Introduction
Cotton (*Gossypium* spp.), an important fibre crop of global significance, is cultivated in tropical and sub-tropical regions of more than seventy countries in the world [1]. It occupies only 5% of the total cultivable area in India, but consumes more than 55% of the total insecticides used in the country [2]. *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) and *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) are the major pests of cotton among the bollworms which cause significant yield losses ref [3]. The introduction of transgenic Bt cotton hybrids, expressing Cry1Ac and Cry2Ab δ- endotoxins from *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (Bt), has been reported to be highly effective against bollworm complex of cotton. Though Bollgard (BG-I) cotton expressing Cry1Ac is still effective against bollworms, gradually it is being replaced by Bollgard II (BG-II) expressing dual genes (*cry1Ac+cry2Ab*) for integrated resistance management (IRM) [4].
Brinjal, *Solanum melongena* L. is an important vegetable crop and extensively cultivated in all South Asian countries (India, Bangladesh, Nepal and Sri Lanka). It is damaged by more than 36 different insect pests right from nursery to harvest [5]. Brinjal shoot and fruit borer (BSFB), *Leucinodes orbonalis* Guenee (Lepidoptera: Pyralidae) is a major insect pest in commercial cultivation of brinjal [6]. Among the vegetables, brinjal receives most number of sprays, an average of 27 times over a period of eight months and most of these sprays were targeted against BSFB [7]. The loss of fruit yield was reported to be up to 70 per cent in case of severe incidence [8].
Brinjal varieties and hybrids expressing *cry1Ac* gene, developed by M/s Mahyco and public sector Institutes (Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu and University of Agricultural Sciences, Dharwad, Karnataka, India) are in advanced stages of commercial release. Brinjal expressing Bt proteins were found to be very effective against BSFB. The Cry2Aa protein has been found to be more toxic to brinjal shoot and fruit borer than Cry1Ab [9].
In the process of development of Bt transgenic plants, it is imperative to evaluate the efficacy of Cry toxins expressed by transformed plants against target pests using a quick, reliable and sensitive method for the determination of insect resistance as large number of transformed events need to be screened to identify a single best event [10].
Till date a number of bioassay methods (leaf disc bioassay, shoot bioassay, whole plant bioassay etc.) have been employed to study the level of resistance in transformed plants [11, 12, 13, 14]. In cotton putative transformants (T_0) are assessed for insect resistance using part of the

plant only *i.e*. leaf disc or detached leaf and whole plant bioassay method is mainly used for screening T_1 generation plants. Whereas, for screening transgenic brinjal against BSFB resistance mostly shoot and fruit bioassay methods are used to test their resistance to insects [15]. However, these methods are time consuming, often laborious and require a lot of space which make them a hard choice for testing the efficacy of Cry toxins against target pests in greenhouse and large-scale field studies. Therefore, it was felt necessary to develop an assay for early screening of Bt transgenic plants which is simple to perform and at the same time quick and more efficient. Hence, in the present study, seedling bioassay protocol was developed and evaluated the bioefficacy of two Bt cotton hybrids *viz.,* Bollgard I expressing *cry1Ac* and Bollgard II expressing *cry1Ac* and *cry2Ab* genes against neonate larvae of *H. armigera* and *S. Litura* and Bt brinjal expressing and Cry2Aa protein against *L. Orbonalis*.

2. Materials and Methods

2.1 Mass rearing of insect pests

2.1.1 Culturing of *S. litura* and *H. armigera*

S. litura and *H. armigera* larvae of different stages were collected from cotton plants raised in Tamil Nadu Agricultural University (TNAU). The larvae were reared on a chickpea (*Cicer arietinum* L.) based semi synthetic diet, consisting chickpea flour 100 g, yeast 30 g, Wesson's salt mix 7.0 g, methyl paraben (4-methyl para hydroxy benzoate) 2.0 g, sorbic acid 1.0 g, ascorbic acid 3.0 g, agar 13.0 g, Vanderzen vitamin solution (28% solution in distilled water) 8 ml, streptomycin sulphate 40 mg, carbendazim (Bavistin 50%WP) 675 mg, formalin 2.0 ml and water 720 ml [16]. Continuous colonies of *S. litura* and *H. armigera* which were free from any disease were maintained on chickpea-based semi synthetic diet at Department of Agricultural Entomology, Centre for Plant Protection Studies, TNAU, Coimbatore, Tamil Nadu, India. Rearing conditions were 27 ºC ±1 ºC, 80±5% RH, and 16:8 h L: D photoperiod. F_2 generation neonates of *H. armigera* and *S. litura* were used for bioassay studies.

2.1.2 Culturing of *L. orbonalis*

Mass culturing of BSFB (Fig. 1) was done as per the standard procedure described by Rajalakshmi *et al*. [17]. For mass culturing, BSFB damaged fruits harbouring larvae were collected from the farmer's field around Coimbatore at fruiting stage.

The fruits were placed in plastic trays containing Dry River sand in culture room at 25 ± 2 ºC and 75-85% RH. The fully grown larvae that came out of the infested fruits were allowed to pupate in the dry river sand. The pupae were collected, placed in a plastic jar (18 x 20 cm, height x diameter) and covered with muslin cloth for adult emergence. Subsequently, newly emerged adults were paired (one male and one female) after sex identification based on the abdominal characteristics (female is bigger than male, with a bulged abdomen) and confined @ 20 pairs in oviposition cages (18 x 20 cm, height x diameter) with tender brinjal twigs/muslin cloth as oviposition substrate. Cotton swabs dipped in honey solution (10% sugar and 0.1% multivitamin Zincovit drops; Apex Laboratories Ltd., Chennai, India) were provided as adult feed. Flat, creamy yellow, scaly eggs were laid on the leaf surface/muslin cloth after 3 days. The twigs or muslin cloths laden with eggs were placed inside the plastic containers lined with muslin cloth for larval emergence. After 3 to 4 days, the newly hatched neonate larvae were transferred on to potato tubers.

Medium sized young potato tubers were washed well in water and a suspension containing 500 mg l^{-1} of carbendazim and 40 mg l^{-1} streptomycin sulphate. The tubers were slightly peeled using knife in three to four places and neonate larvae at the rate of 30 to 40 larvae per tuber were placed over the peeled region. The tubers were placed individually over dry river sand, kept in a plastic container (15 x 5 cm diameter x depth) covered with muslin cloth. The matured larvae came out of potato and pupated in dry sand medium. Subsequent to pupation, the method described earlier for field collected brinjal fruits was adopted for adult emergence and oviposition. Newly hatched first instar larvae (neonates) of F_2 generation were either used for bioassay or transferred to peeled regions of potato tubers and reared for many generations in the laboratory conditions for further bioassay experiments.

2.2 Sources of transgenic plant materials

The transgenic cotton materials used in this study were i) seedlings of commercially available hybrids *viz.,* Bollgard I (NCS 207) expressing Cry1Ac protein and Bollgard II (RCH 2) expressing Cry1Ac and Cry2Ab proteins, protein expression confirmed by Enzyme-linked immunosorbent assay (ELISA) (data not shown) ii) PCR positive T_1 progeny seedlings of Co2 brinjal (CS-Co2-15,CS-Co2-16, CS-Co2-19, CS-Co2-35, CS-Co2-46 and CS-Co2-49) and EVB (CS-EVB-8) (Ellavambadi brinjal) cultivars expressing synthetic *cry2Aa* gene (Fig. 2) and Co2-*Bt* brinjal expressing *cry1Ac*. All brinjal genotypes with Bt genes were developed at Centre for Plant Molecular Biology & Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. Bt *cry2Aa* gene used in the present study for development of Bt brinjal was isolated by Dr. V. Udayasuriyan (Department of Plant Biotechnology) and his team and subsequently codon-optimized by Gupta (2005).

2.3 Seedling bioassay in cotton with *H. armigera* and *S. litura*

The experiment was conducted at transgenic greenhouse (28 ± 1 ºC, 70±5% RH with a photoperiod of 16:8h L: D photoperiod) of Centre for Plant Molecular Biology and Biotechnology, TNAU, Coimbatore during February-March 2012. The seeds of Bollgard I and Bollgard II hybrids and their corresponding isogenic cotton genotype were sown in alternative rows in pro trays (size of 53 x 28 cm, length x width) (Fig. 3). Eight replications (each replication consisted of 10 seedlings) were used for each transgenic cotton hybrid (BG-I and BG-II). A control (Coker 312) with eight replications was also included.

Ten day old seedlings *i.e.,* seedlings having two true cotyledonary leaves were used for bioassay. A single neonate larva of *S. litura* and *H. armigera* was gently transferred with the help of a moistened camel hair brush onto each leaf. The mortality of larvae and leaf-area damage were recorded after 24 and 48 hours of release and converted as per cent larval mortality and leaf area damage.

2.4 Seedling bioassay in Bt brinjal with *L. orbonalis*

The T_1 seeds of Co2 and EVB cultivars expressing *cry2Aa* gene, Co2-Bt brinjal expressing *cry1Ac* gene (as positive control) and respective negative control plants were sown in protrays in alternative rows in greenhouse. Each treatment

was replicated thrice by dividing a line of 30 plants into three, with each replication consisting of 10 seedlings. After 30 days, with the help of a moistened camel hair brush two neonate larvae per seedling were released on growing shoot bud. The observation on number of seedlings showing drooping symptom was recorded after 96 hours of release and was converted as per cent seedling damage.

2.5 Statistical analysis

For analysis of variance (ANOVA), the per cent values were transformed to their respective arcsine values. Data on per cent larval mortality and leaf area consumed were statistically analysed as completely randomised design (CRD) and the least significant differences (LSD) between means were calculated by Duncan's multiple range test (DMRT) at 5 per cent (P=0.05) probability level [18] using the IRRISTAT Version 3.1 (International Rice Research Institute, Philippines).

3. Results and Discussion

3.1 Bt cotton seedling bioassay with neonate *S. litura*

Results of the bioassay with *S. litura* on seedlings of BG-I and BG-II Bt cotton are summarized in the Table 1. Clear indications of toxicity became apparent after the second day of feeding. Larvae that fed on Bt plants became sluggish and turned black. These were the typical symptoms of Bt toxicity (Fig. 4). The mean larval mortality of neonates that fed on the BG-II expressing two proteins was 99.37 per cent after 24 and 48 hours of bioassay and the seedlings were completely resistant to neonate larvae. However, no mortality was observed in BG-I and control (non-Bt cotton), and the larvae reached second instar. Observation on leaf area damage indicated that the leaf area consumed by *S. litura* neonates was low on BG-II (1.50%) after 24 and 48 hours of bioassay. After 48 hours of larval release, leaf area damage was 12.50 and 15.62 per cent in BG-I and control seedlings, respectively. The results revealed that the feeding damage on BG-I and control seedlings was more prominent than on BG-II seedlings and it was visible to the naked eye.

3.2 Bt cotton seedling bioassay with neonate *H. armigera*

Results of the insect bioassays with neonate larvae of *H. armigera* demonstrated a significant level of insect protection by the seedlings of transgenic Bt cotton hybrids (BG-I and BG-II). Mortality observations are presented in Table 1. At 48 hours of release, BG-II recorded 81.25 per cent mortality which was significantly higher than BG-I (75.62%). The larvae that fed on the control seedlings were alive after 48 hours and caused considerable damage to the cotton leaves. After 48 hours of feeding, 8.12 and 9.25 per cent leaf damage was recorded in BG-II and BG-I, respectively while the damage was 16.75 per cent in control plants.

3.3 Bt brinjal seedling bioassay with neonate *L. orbonalis*

Insecticidal effect of Cry2Aa protein against neonate BSFB was assessed at the seedling stage of T_1 progenies. The progenies showed significant resistance against neonates of BFSB. A significant difference in seedling damage was found among the transgenic lines. In control plants, seedlings started drooping within 24 hours of release of larvae, resulted in a wilted shoots and shedding of leaves at 48 hours which were the typical symptoms caused by BSFB (Fig. 5). The present study was unable to record the BSFB larval mortality as the larvae fed and died inside the stem, and it was difficult to locate larvae that were concealed inside the seedlings.

To test the effectiveness of the Cry2Aa protein in seedlings, six T_1 Co2 lines, (CS-Co2-15, CS-Co2-16, CS-Co2-19, CS-Co2-35, CS-Co2-46 and CS-Co2-49) and one EVB (CS-EVB-8) line were subjected to seedling bioassay. The line CS-Co2-46 showed the lowest seedling damage of 53.33% after 96 hours of release of neonates (Table 2; Fig. 6). Two Co2 lines CS-Co2-15 and CS-Co2-19 were on par with each other and recorded 60.00 per cent damage. On the other hand, the non-transgenic Co2 control plants recorded 86.67 per cent damage. In Co2-*Bt* brinjal no (0.00%) damage was observed. In case of EVB, CS-EVB-8 recorded 49.05 per cent, while in control 87.06 per cent damage was observed.

Several bioassay techniques have been used in the past to evaluate the transgenic plants of maize, cotton and potatoes for resistance to insect pests at the initial stages of screening [19]. These techniques typically used either leaf bioassays (leaf disc and detached leaves from young plants) which typically have the highest Bt protein expression in leaf tissues [20] or whole plants bioassays following culture under greenhouse conditions. Pollen and root bioassays were also used for screening of Bt transgenic maize against neonate larvae of European corn borer and corn rootworm larvae, respectively [19].

Leaf disc bioassay method includes leaf discs of appropriate diameter excised from young leaves and placed on agar containing antimicrobials in a Petri plate with moistened filter paper [11, 13]. In leaf disc bioassay, desiccation of leaf discs takes place and the leaves from the transformed plants need to be replaced with a fresh leaf at least once in two days, which would be a constraint for researchers who assay young plants having four to five leaves [10, 21, 4]. This method is laborious because cutting leaf discs of appropriate size consumes more time, besides browning of leaves occurs at cut ends due to accumulation of some secondary metabolites like phenolics. These phenolics may interfere with actual toxicity of Cry proteins and the observation may produce inaccurate results. [22] Observed that there were many physiological changes in plants that contribute to plant-toxin interactions in cotton. Another drawback of the assay was the degradation of Cry protein (Cry1Ac) after detachment of the leaf tissues from the plant leading to inaccurate results [13]. Similarly, [23] standardized a bioassay technique which consisted of a leaf bit measuring about 10 mm^2 taken from the transformants to be tested, placed on agar in a transparent glass vial. The vial was then sealed tightly with sterile cotton to avoid contamination. Though this technique is reliable, obtaining leaf bits and measuring mortality individually is laborious.

Earlier [24] and [12] standardized a detached leaf bioassay method by keeping fully expanded cotton and trifoliate pigeonpea (*Cajanus cajan* (L.) leaves in plastic cups (250 ml) with the petiole inserted into the agar substratum. Though this technique used 30 to 45 day old plants and kept the leaves in a turgid condition for one week, screening of large number of plants was laborious and required lot of laboratory space. For screening cotton plants using this method, the plants were raised on sterilised mixture of black soil, sand and farmyard manure (2:1:1). The soil was filled into medium sized pots of 30 cm diameter and 30 cm depth and each genotype required 5 such pots [12]. Hence, this method has drawbacks like more input and maintenance cost for potting and maintaining the plants under greenhouse conditions. Moreover we need to wait until 45 days for testing plants for insect resistance.

For insect assays with whole plants, transgenic greenhouse-grown cotton lines (T_1 progeny) producing Cry proteins were exposed to neonate larvae. Even though, this technique was

found to give better results overcoming the problems associated with detached leaf and leaf disc bioassay, major limitations of this technique are that it takes long time (2-3 months) to evaluate the efficacy of Cry toxins against target pest and requires large area to raise transgenic plants in pots under greenhouse conditions [25, 13] which in turn increases the labour requirement and maintenance cost. [20] Conducted laboratory bioassays using different plant parts (leaves/squares/flowers/bolls) of Bt and non-Bt cotton hybrids with *H. armigera*. In order to perform these bioassays, the transgenic plants need to be grown in the green house until they reach the flowering or fruiting stage (3 to 4 months). In the present study, we have developed a novel seedling bioassay method to assess the resistance of transgenic brinjal plants expressing Cry2Aa protein to BSFB neonates. One month old seedlings of transgenic and non-transgenic genotypes of Co2 and EVB expressing Cry2Aa were evaluated for resistance to feeding by neonates. The results of seedling bioassay indicated that there was significant difference in seedling damage among transgenic and control plants, with control plants exhibiting more damage. This method is very simple, cheap, and rapid and requires less space and time (4 days) to screen the transgenic plants for insect resistance (Table 3). Seedling bioassay method used in the present study was effective in identifying those lines with intermediate to high level of resistance.

Seedling bioassay with Bt cotton plants against *S. litura* neonates revealed that the larvae that fed on control and BG-I were alive and caused significant leaf damage (6.25-8.75%) to seedlings indicating that BG-I was ineffective against *S. litura*. Whereas, BG-II caused 99.37 per cent mortality after 48 hours of insect release indicating that the cotton expressing two proteins (Cry1Ac and Cry2Ab) was effective against *S. litura*. The results of the current experiment indicated that the seedling bioassays with Bollgard II cotton provided significant protection against *S. litura*. These findings corroborate with the results obtained by Adamczyk *et al*. (2008) [21] and Akin *et al*. (2010) [26] with *Spodoptera frugiperda* (J.E. Smith), *Spodoptera exigua* (Hubner), respectively. Kesey and Kerns (2010) [27] also reported that the seedlings (at two true- leaf stage) of Bollgard II and Widestrike *Bt* cotton were highly resistant to neonate salt marsh caterpillars, killing 100 per cent with no visible damage after 3 days of exposure.

Seedling bioassay was also carried out using *H. armigera*, which is the major target pest of Bt-cotton in India, China and Australia. Results on larval mortality indicated that this technique is mainly useful for screening transgenic plants against leaf feeders and internal borers. In contrast to that, *H. armigera* is primarily a bollworm and prefers feeding on fruiting parts in the late instar stage and feeds on foliage only in early instar stage [28]. Leaf bioassays using leaf disc and detached leaf bioassay are useful for screening putative transformants (T_0 plants) against test insects whereas, for screening of segregating progenies seedling bioassay is the most effective method. Insect bioassays with seedlings are more appropriate where the test insect is a leaf feeder, stem or shoot borer. However, the damage in the reproductive part may not be assessed appropriately with this method.

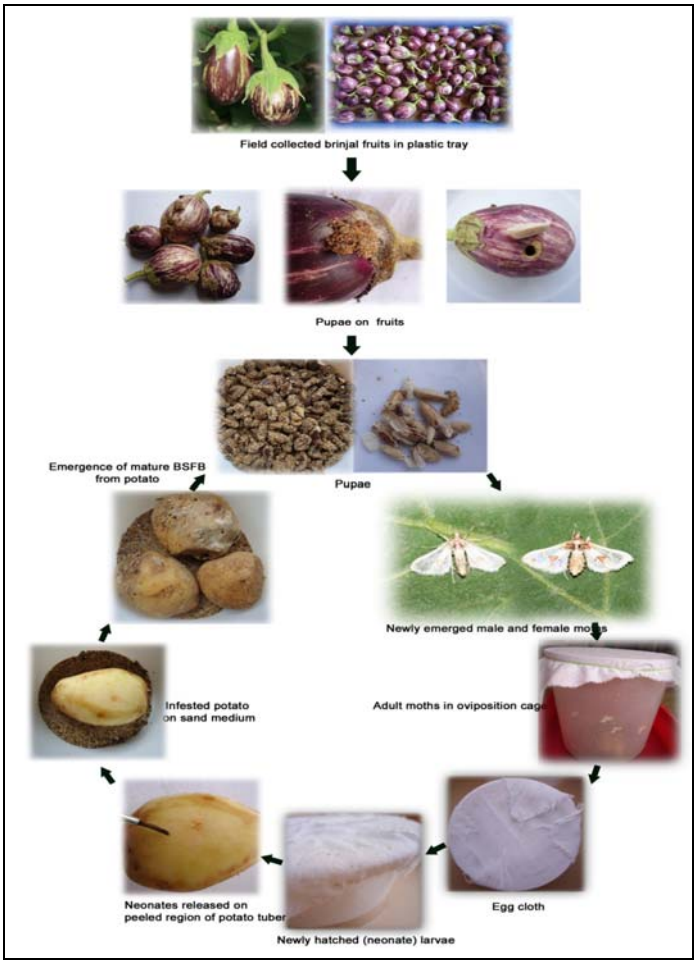


Fig 1: Mass culturing of brinjal shoot and fruit borer

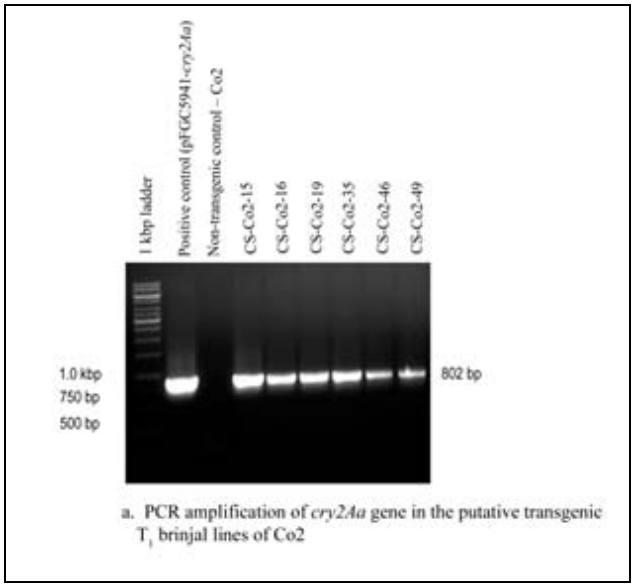


Fig 2: PCR amplification of *cry2Aa* gene in T_1 progenies of transgenic brinjal plants

Fig 3: Bt cotton bioassay using 10-day old seedlings

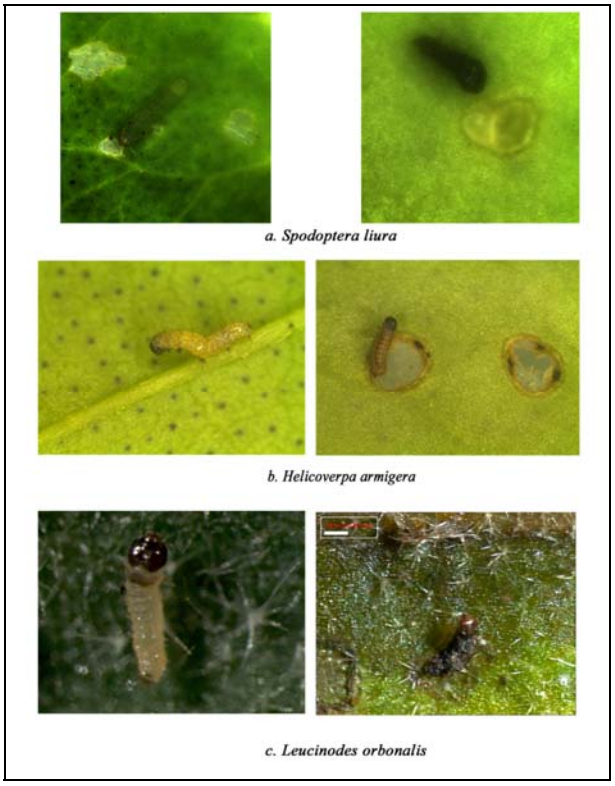
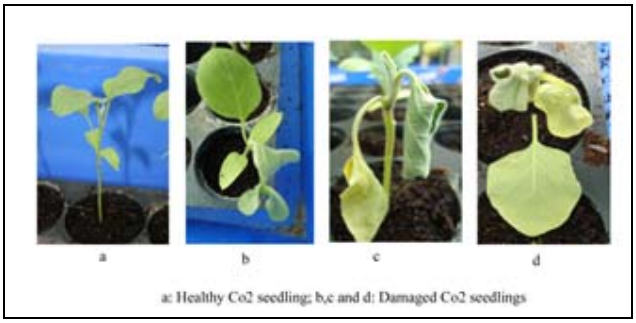


Fig 4: Comparison between control and Bt infected larvae



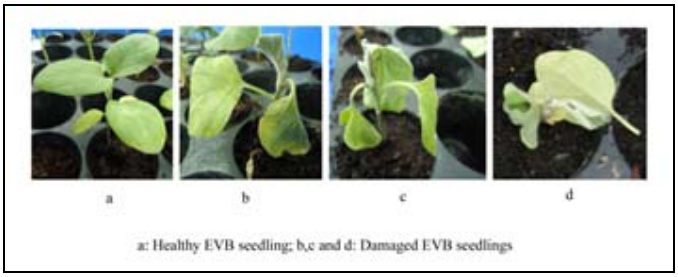


Fig 5: Different stages of damage symptoms caused by BSFB neonates on Co2 and EVB seedlings

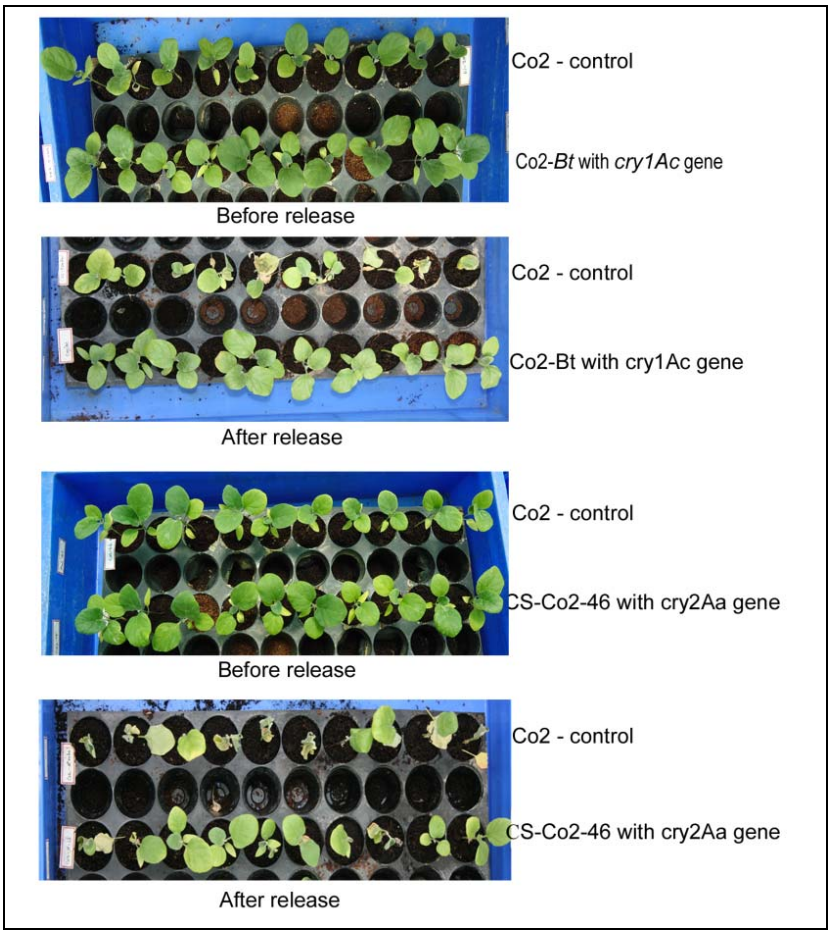


Fig 6: Screening of Bt brinjal seedlings expressing Cry2Aa protein against BSFB

Table 1: Seedling bioassay with neonate *Spodoptera litura and Helicoverpa armigera* on BG-I and BG-II Bt cotton hybrids

Genotypes	*Spodoptera litura*				*Helicoverpa armigera*			
	Larval mortality (%)*		Leaf damage (%)*		Larval mortality (%)*		Leaf damage (%)*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Bollgard I	0.00 (0.07) [b]	0.00 (0.07) [b]	6.25 (14.30) [b]	12.50 (20.67) [b]	41.25 (39.96) [b]	75.62 (60.43) [b]	8.56 (17.01) [b]	9.25 (17.69) [b]
Bollgard II	99.37 (88.38) [a]	99.37 (88.38) [a]	1.50 (6.93) [a]	1.50 (6.93) [a]	52.50 (46.44) [a]	81.25 (64.38) [a]	7.25 (15.61) [a]	8.12 (16.54) [a]
Control	0.00 (0.07) [b]	0.00 (0.07) [b]	8.75 (17.06) [c]	15.62 (23.20) [c]	0.00 (0.07) [c]	0.00 (0.07) [c]	12.50 (20.70) [c]	16.75 (24.15) [c]

*Mean of eight replications

Figures in parentheses are arcsine transformed values

Means followed by small letter(s) in common within columns (lower case letters) are not significantly different at 5% level by DMRT ($P<0.05$; Duncan"s test)

Table 2: Evaluation of seedlings of T_1 progenies expressing Cry2Aa protein for resistance to feeding by neonate BSFB

Brinjal T_1 lines	Per cent seedling damage (96 h observation) *
CS-Co2-15	60.00 (51.93) [ab]
CS-Co2-16	78.73 (62.72) [ab]
CS-Co2-19	60.00 (51.14) [ab]
CS-Co2-35	76.40 (61.00) [ab]
CS-Co2-46	53.33 (47.21) [a]
CS-Co2-49	62.62 (52.55) [ab]
Co2-control	86.67 (72.29) [b]
CS-EVB-8	49.05 (44.44) [a]
EVB-control	87.06 (69.28) [b]
Co2- Bt *cry1Ac* (positive control)	0.00

* Mean of three replications.

Figures in parentheses are arcsine transformed values.

Means followed by small letter (s) in common within columns (lower case letters) are not significantly different at 5% ($P<0.05$; Duncan"s test)

Table 3: Comparison of various bioassay methods used for screening of transgenic brinjal and cotton

Particulars (approximate)	Seedling bioassay	Leaf disc/ detached leaf bioassay	Whole plant bioassay	Fruiting part bioassay
Time requirement (i) cotton (ii) brinjal	 7-10 days 25- 30 days	 30-45 days 50-60 days	 60-70 days 60-80 days	 80-90 days 90-120 days
Space requirement	1.8 meter for screening 100 seedlings	20 meter laboratory space	100 meter for 100 plants	100 meter for 100 plants
Man hours requirement	10 h One labour for 10 days	60-90 h One labour till the end of bioassay for maintenance of plants. One technical person for making leaf discs of appropriate size	120-160 h One labour till the end of bioassay for maintenance	120-160 h One labour till the end of bioassay for maintenance
Pot requirement	4 Protrays (53 x 28 cm, length x width) of 90 cells each	100 pots (23 cm x 29 cm, height x diameter) needed	100 pots (23 cm x 29 cm height x diameter) needed	100 pots (23 cm x 29 cm height x diameter) needed
Potting mixture	Vermicompost (1 kg/ 100 seedlings) is used for initial establishment of plants	Vermicompost (15-20 kg/ 100 plants) is used for initial establishment of plants	Vermicompost (15-20 kg/ 100 plants) is used for initial establishment of plants	Vermicompost (15-20 kg/ 100 plants) is used for initial establishment of plants
Fertilizer application	-	Potassium nitrate 2 kg + Calcium nitrate 2 kg + AQUAFERT 2 kg	Potassium nitrate 2 kg + Calcium nitrate 2 kg + AQUAFERT 2 kg	Potassium nitrate 2 kg + Calcium nitrate 2 kg + AQUAFERT 2 kg
Total cost involved (approx.)	Rs.250	Rs.8141	Rs.8141	Rs.8141

4. Conclusions

In conclusion, the present study demonstrated that the seedling bioassay technique can be used effectively for rapid and early screening of resistant events from large number of transformants. The present method ensures and shortens the time needed for screening of Bt plants. The seedling bioassay was found to be a reliable technique for screening of transgenic plants against leaf feeders and internal borers *viz*., *S. litura,* and BSFB. This method can also be used for evaluation of putative transgenic plants for resistance to insects in the early segregating generations.

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