A new observation on feeding behaviour of pink bollworm and its application in screening *Bt*-resistant population

A. L. Reyaz¹ · N. Balakrishnan¹ · V. Udayasuriyan¹

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Abstract

The pink bollworm (PBW), *Pectinophora gossypiella* (Saund.) does not feed on leaves in natural circumstances. We made an attempt with the first instar larva (5 days old) and found it eats the leaf in the absence of other foods. Utilizing this new feeding behaviour, a simple methodology was developed for screening of resistance in PBW against *Bt* cotton plants. The PBW collected from BGII *Bt* cotton fields (BGII-resistant population) and NBAIR culture (susceptible population) were reared under laboratory conditions for two generations. Laboratory reared 5-day old larvae of PBW were released on cotton leaf discs individually for screening. The BGII-resistant and susceptible larvae fed an average leaf area of 75.52 ± 16.68 and 5.95 ± 0.93 mm² with survival rate of 90 and 4% respectively, in BGII *Bt* cotton leaves. Whereas in case of non-*Bt* cotton (MCU13), the BGII-resistant and susceptible larvae consumed average leaf area of 114.84 ± 23.70 and 116.80 ± 24.14 mm² with survival rate of 93.34 and 95.33%, respectively. In addition to the screening process, the larval survivors were transferred to an artificial diet after 7 days of experiment and observed up to their emergence as adults. To confirm the development of resistance in PBW, sequencing of larval DNA amplicons was carried out and it revealed mutation in the cadherin gene of the BGII-resistant PBW population. Hence, the detached leaf bit feeding assay described here could be used in a simple manner for screening of resistance developed by PBW against *Bt* cotton and also for evaluating *Bt* cotton plants for their inherent resistance to PBW. This method could also be used for studying toxicity of *Bt* isolates by coating spore-crystal mixture on non-transgenic cotton leaf discs.

Keywords *Pectinophora gossypiella* · Bioassay · Resistance · *Bt* cotton · Cadherin

Introduction

Cotton is an important commercial crop and insect-resistant transgenic *Bt* cotton is cultivated to mitigate the damage caused by lepidopteran insect pests, viz., American bollworm (*Helicoverpa armigera*), spotted bollworm (*Earias*

vittella), pink bollworm (*Pectinophora gossypiella*), and the tobacco caterpillar (*Spodoptera litura*). The benefits of *Bt* cotton include considerable reduction of insecticide usage against lepidopteran insect pests and increased yield due to control of bollworms (Carpenter 2010; Lu 2012). The area planted to biotech upland cotton globally in 2016 was 22.3 million hectares (ISAAA 2016). Unfortunately, the benefits of such *Bt* technology can be cut short by the evolution of resistance by pests. Gene pyramiding strategy to combine Cry1Ac and Cry2Ab has proven to be effective in delaying the development of resistance to *Bt* toxins (Jackson et al. 2003; Zhao et al. 2003).

In 2014–2015, approximately 95% of the area producing cotton in India was sown with transgenic hybrids containing the *cry1Ac* gene (Bollgard I) or two *cry* genes, *cry1Ac* and *cry2Ab* (Bollgard II) (Singh et al. 2016). Since the introduction of *Bt* cotton in India, the cotton production area has increased from 7.7 to 10.8 million hectares and fiber

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✉ V. Udayasuriyan
udayvar@yahoo.com
A. L. Reyaz
sweetreyaz@gmail.com
N. Balakrishnan
bala8775@gmail.com

¹ Department of Plant Biotechnology, CPMB&B, Tamil Nadu Agricultural University, Coimbatore 641 003, India

production has increased from 13.6 to 33.8 million bales (ISAAA 2016).

Field populations of the pink bollworm in the United States have remained susceptible to *Bt* toxins Cry1Ac and Cry2Ab, but field-evolved practical resistance to *Bt* cotton producing Cry1Ac has occurred widely in India (Monsanto 2010; Fabrick et al. 2014, 2015). The PBW may also have evolved resistance to the dual-toxin *Bt* cotton (Bollgard II) in India, as significant damage by PBW was noticed (Kranthi 2015) and has crossed ETL in Gujarat, Andhra Pradesh, Telangana, Maharashtra and Karnataka [ICAR-AICRP (Cotton) Annual Report, 2016–2017]. Cadherin proteins bind *Bacillus thuringiensis* (*Bt*) toxins in lepidopteran midguts (Morin et al. 2003). Any modifications of target sites reduce or eliminate binding of *Bt* toxins in homozygous resistant individuals. Mutations in cadherin gene of PBW have been reported to be associated with the resistance towards the *Bt* proteins (Tabashnik 1994; Fabrick et al. 2014).

Screening for susceptibility of the PBW towards the *Bt* proteins is performed by diet incorporation method (Muralimohan et al. 2009; Fabrick et al. 2014). This requires importation of expensive commercial *Bt* protein(s) and its proper incorporation in artificial diet. In order to screen the PBW for resistance against *Bt*-cotton, an easy and economical method is needed. We tried to feed neonates on leaf discs but none could survive. This observation was similar to what has been reported for pink bollworm earlier, in which it does not feed on vegetative parts (other than on boll/square to access seed/floral components). In fact, the early emergence of pink bollworm before cotton bolls are available results in “suicide generation”, in which the emerging generation ultimately dies because larvae do not feed on leaves/stems of cotton (Bariola 1978). Chapman et al. (1960) defined that suicidal emergence was that emergence that occurred 3 or 4 days prior to the advent of 1st squares on the cotton. However, in this study for the first time we found that 5-day old pink bollworm larva (first instar) feeds on cotton leaf disc which is undocumented previously. Here, we have reported for the first time usage of cotton leaf disc for determining susceptibility of PBW towards the *Bt* proteins expressed in transgenic cotton plants.

Materials and methods

Pectinophora gossypiella (NBAIL-MP-GEL-02) culture is maintained under laboratory conditions for a long period of time at Indian Council of Agricultural Research-National Bureau of Agricultural Insect Resources (ICAR-NBAIR), Bengaluru, India. The eggs of PBW were procured from NBAIR, Bengaluru. We also collected PBW population from *Bt* cotton field. Before sampling in Bollgard II (BGII) *Bt* cotton field, 25 plants were selected per field randomly

and presence of Cry1Ac and Cry2Ab were confirmed by Quick strips (Cat. No. AID 026, Amar Immunodiagnostics, Hyderabad, India). A total of 1422 mature green bolls were collected from infested BGII *Bt* cotton fields in Kurnool district, Andhra Pradesh, India, during 2 October 2016–6 October 2016, packed in cotton bags and brought to the laboratory (online resource 1). The bolls were cut opened with a knife and a total of 634 larvae and 28 pupae of PBW were obtained and this culture (KAP) was expected to be resistant to BGII cotton. Both the susceptible and resistant cultures (NBAIR and KAP) were maintained on artificial diet (Dhara Jothi et al. 2016) in individual sterile glass injection bottles (5 cm long and 2.4 cm dia) under laboratory conditions at 28 °C, RH 60±5% and 12 h light and 12 h dark photoperiod. The individuals from PBW cultures were sexed during larval stage based upon the presence of a pair of dark coloured testis on the dorsal side of the 7th to 8th abdominal segments of male which is absent in female. Pupae were collected, surface-sterilized in 0.05% sodium hypochlorite for 2 min followed by several quick rinses in distilled water, dried on paper towels and were kept in adult emergence cages in equal male and female ratio.

The adults were fed with 10% sugar solution (supplemented with the vitamin solution—1 ml/500 ml) provided in a ball of absorbent cotton placed in a small glass dish inside the oviposition cage. A bouquet of cotton leaves/twigs was kept inside cage. Eggs laid by moths on leaves were transferred to a plastic container (25 cm height and 7.5 cm diameter) and covered by a muslin cloth. The containers were examined daily and neonates were transferred to artificial diet using a fine hair brush. After completions of two generations, the larvae were used for the study.

Seeds of BGII *Bt* cotton (RCH2 *Bt* hybrid from Rasi Seeds Pvt. Ltd. Attur, TN, India) and non-*Bt* cotton MCU13 (a TNAU variety) were sown and ten plants were maintained in transgenic greenhouse following recommended cultivation practices except insecticide sprays. Expression of Cry1Ac and Cry2Ab in leaf samples was checked using Quick strips (Cat. No. AID 026, Amar Immunodiagnostics, Hyderabad, India) and the concentration of Cry2Ab in leaf samples was analyzed on 66 DAS using QuantiPlate kit (EnviroLogix, USA) as per the manufacturer's instructions.

Third top most leaves from the BGII and non-*Bt* MCU13 cotton variety were used for the bioassay purpose. Small leaf discs of 1.4 cm diameter were cut from the leaf, washed with distilled water and allowed to dry on tissue paper to remove the excess moisture. These were placed individually in a Petri dish (90 mm dia.) having a moist Whatman filter paper (Whatman™ 1) to avoid desiccation. Active 5-day old PBW larvae (NBAIR culture and KAP culture) were taken from the artificial diet with a fine hair brush, starved for 2 h, and released onto the leaf disc in a laminar air flow chamber. A total of 150 larvae from resistant and susceptible

populations were used for each bioassay experiment. One larva was released on each leaf disc, allowed to feed for 7 days and observations were recorded. If the leaf disc had been eaten fully it was replaced by a fresh one. The leaf area fed by the larvae was measured using graph sheets. Larvae which survived on the leaf discs after 7 days were transferred onto the artificial diet (Dhara Jothi et al. 2016) and allowed to grow further.

The genomic DNA was extracted from bioassayed PBW (larval survivors) using cetyl trimethyl ammonium bromide (CTAB) method. The quality and quantity of DNA were assessed using NanoDrop 1000[®] spectrophotometer (Thermo Scientific, USA) and agarose gel. PCR was carried out in a thermocycler (Eppendorf, Germany) to amplify insect genomic DNA using cadherin gene specific primers viz. 171PgCad5 and 172PgCad3 described by Fabrick et al. (2014). InsTAclone[™] PCR Cloning Kit (Fermentas Life Science, Mumbai, India) was used for cloning of gel purified PCR products. The PCR products were ligated in pTZ57R/T vector as per instructions in the user manual and transformed into *Escherichia coli* DH5 α . Putative positive clones were identified by blue-white screening and colony PCR. The recombinant plasmid DNA was extracted from the putative clones using EZ-10 spin column Plasmid isolation kit (Bio Basic INC, Canada) and the existence of the cloned gene was further confirmed by PCR using gene specific primers. The recombinant pTZ57R/T-Cad plasmids were sequenced using M13 primers by automated DNA sequencer

(ABI 3730xl) at AgriGenome Labs Pvt Ltd, Bengaluru. The forward and reverse gene sequences were assembled using BioEdit program (Hall 1999) and analyzed using the Clustal Omega software tool (Sievers et al. 2011). The sequences were deposited into GenBank.

Results

Before starting the bioassay experiment, presence of Cry1Ac and Cry2Ab in the leaves of Bollgard II *Bt* cotton plants was confirmed by lateral flow assay strips. The concentration of Cry2Ab in BGII cotton plant leaf samples was found to be 19.10 ± 0.56 $\mu\text{g/g}$ in fresh leaf tissue. The proteins Cry1Ac and Cry2Ab were not detected in non-*Bt* cotton, MCU13 (online resource 2).

After 7 days of bioassay experiment, the larvae of resistant population fed an average leaf area of 75.52 ± 16.68 and 114.84 ± 23.70 mm² and recorded a survival rate of 90 and 93.34% in BGII *Bt* and non-*Bt* (MCU13) cotton leaves respectively. The larvae of susceptible population consumed an average leaf area of 5.95 ± 0.93 and 116.80 ± 24.14 mm² and recorded a survival rate of 4.00 and 95.33% in BGII *Bt* and non-*Bt* (MCU13) cotton leaves (Fig. 1). After 7 days, the survived larvae of resistant population on the leaf discs of BGII *Bt* cotton (90%) were transferred to artificial diet. Seven well-grown larvae were used for molecular work. The larvae reached pupal stage in 23.5 ± 2.44

Fig. 1 *Pectinophora gossypiella* bioassay using cotton leaf disc

days after hatching. The pupal period was 7.53 ± 1.03 days with average pupal weight of 13.34 ± 0.87 mg. Out of 143 ($150 - 7 = 143$) larvae, 96 reached the pupal stage (i.e.) 67.13% and 88 reached adult stage (i.e.) 61.53%. The survived larvae of resistant population on the leaf discs of non-*Bt* cotton (93.34%) were transferred to artificial diet and reached pupal stage in 22.56 ± 2.55 days after hatching and the pupal period was 7.34 ± 0.70 days with an average pupal weight of 18.46 ± 1.93 mg. The adult emergence was 62.66%. Likewise, the survived larvae (retarded growth) of susceptible population on the leaf discs of BGII *Bt* cotton (4.00%) were transferred to artificial diet and attained the pupal stage (2% only) in 38.16 ± 1.75 days after hatching. The pupae were very small and malformed with an average weight of 7.33 ± 0.57 mg and there was no adult emergence. The survivors of the same population on the leaf discs of non-*Bt* cotton (95.33%) were transferred to artificial diet. They reached the pupation stage in 21.92 ± 2.28 days after hatching. The pupal stage was 7.23 ± 0.44 days and weighed 18.49 ± 1.93 mg per pupa. The adult emergence was 69.38% (Table 1).

Based on survival rates, it was concluded that NBAIR population is susceptible and field-collected population is resistant to BGII. We analyzed DNA from three NBAIR PBW (non-*Bt* leaf disc) and seven BGII survivors from KAP culture. PCR for resistance related receptor (cadherin gene) with the genomic DNA extracted from bioassayed PBW (larvae) samples from resistant and susceptible populations gave an expected amplicon size of approximately 575 bp, except one of the insect samples in resistant population which gave an extra prominent band of approximately 700 bp (Fig. 2). Gel purified products were ligated into pTZ57R/T and transformed in *E. coli* DH5 α .

Fig. 2 Amplification of partial cadherin gene in NBAIR and KAP larvae of PBW. Lane 1: 100 bp marker; lane 2: Negative control (water); lane 3–5: NBAIR larvae; lane 6–12: KAP larvae

PCR of isolated recombinant plasmids from the putative transformants with gene specific primers gave amplicons of approximately 575 and 700 bp. DNA sequencing results showed that all amplified products (575 and 697 bp) correspond to the targeted site of the partial cadherin gene. In three individuals of NBAIR (susceptible) culture, DNA was found intact in this cadherin gene region (Accession no. KY798128) However, in resistant culture, one sample has shown severely disrupted cadherin gene with insertion of 126 bp in exon region and deletion of 1 bp in intron region (Accession no. KY798127).

Table 1 Screening of *Pectinophora gossypiella* for resistance towards Bt proteins

Particulars	NBAIR-BGII	NBAIR-MCU13	KAP-BGII	KAP-MCU13
No. of larvae used	150	150	150	150
Total leaf area fed (7th day) (mm ²)	892.50	17520.40	11328.00	17225.27
Average leaf area fed by each larva (mm ²)	5.95 ± 0.93	116.80 ± 24.14	75.52 ± 16.68	114.84 ± 23.70
Survival percentage (7th day)	4.00	95.33 ^a	90 ^b	93.34
Larval period (days)	38.16 ± 1.75	21.92 ± 2.28	23.5 ± 2.44	22.56 ± 2.55
Pupal period (days)	Not hatched	7.23 ± 0.44	7.53 ± 1.03	7.34 ± 0.70
Average weight of pupa (mg)	7.33 ± 0.57	18.49 ± 1.93	13.34 ± 0.87	18.46 ± 1.93
Pupal percentage ^c	2.00	72.10	67.13	70
Emergence percentage ^c	Nil	69.38	61.53	62.66

^aThree well grown larvae were used for molecular work

^bSeven well grown larvae were used for molecular work

^cPercentage was calculated with reference to larvae initially used for experiment (i.e. 150 nos.) except for NBAIR-MCU13 and KAP-BGII where it was calculated with reference to 147 ($150 - 3 = 147$) and 143 ($150 - 7 = 143$) respectively

Discussion

Pectinophora gossypiella does not feed on leaves in natural circumstances. We made an attempt with 5 days old first instar larva and found it eats the leaf in the absence of other foods, grows and reaches up to fourth instar on leaf disc. Thus, it was conceived, leaf could be utilized for screening purpose of resistance towards *Bt* proteins in transgenic cotton. We tested our hypothesis with a susceptible (NBAIR culture) and a resistant population (collected from infested BGII cotton fields). The bioassay results revealed that NBAIR culture feeds non-*Bt* cotton leaf disc (with an average area of $116.80 \pm 24.14 \text{ mm}^2$) and proceeds into other instars with 95.33% survival rate. However, the same culture could not feed on the BGII leaf disc leading to death of 96% larvae and the survivors showed the very retarded growth (online resource 3 and 4). Only a few bites were visible on each leaf disc (approximate leaf area fed was $5.95 \pm 0.93 \text{ mm}^2$). This indicates larvae had stopped feeding immediately after release due to the action of Cry proteins which are known to cause lyses of midgut epithelial cells and osmotic imbalance and eventually leads to the death of the insects (Knowles et al. 1987; Hofmann et al. 1988). In case of the resistant population, 93.34% survived on non-*Bt* cotton leaf disc with average feeding of $114.84 \pm 23.70 \text{ mm}^2$, whereas on the BGII leaf disc 90% survived on seventh day after release with an average feeding area consumption of $75.52 \pm 16.68 \text{ mm}^2$ only, which was less as compared to larvae feeding on the non-*Bt* cotton leaf disc. Possible reason for this could be the fitness cost of resistance from the *Bt* proteins (Sosa-Gómez and Miranda 2012).

The Cry proteins of *B. thuringiensis* bind to the specific receptor molecules of epithelial cells in the midgut. One of major classes of receptors is the cadherin-like receptors (Pigott and Ellar 2007). Caccia et al. (2010) reported an alteration in the receptor is responsible for field-isolated resistance to *B. thuringiensis* insecticidal proteins in two *Helicoverpa* species. Morin et al. (2003) reported association of three cadherin alleles with resistance to *B. thuringiensis* in pink bollworm. Each of the three resistance alleles had a deletion expected to eliminate at least eight amino acids upstream of the putative toxin-binding region of the cadherin protein. Recently, DNA sequencing of pink bollworm by Fabrick et al. (2014) derived from resistant field populations in India revealed presence of new eight severely disrupted cadherin alleles associated with resistance to Cry1Ac. These eight alleles were found to produce a total of 19 transcript isoforms, each containing a premature stop codon, a deletion of at least 99 base pairs, or both. Hence, we also used PCR method to screen the genomic DNA (gDNA) of pink bollworm for the presence

of mutated cadherin alleles in population which survived on the BGII leaf disc. DNA sequencing of the amplified products revealed disrupted cadherin gene in one of the seven insect samples of field collected resistant population. An insertion of 126 bp in the coding region and deletion/base substitution of few base pairs in the non-coding region were found in the particular sequenced region of the gene. Cadherin DNA sequences of NBAIR culture in particular region were found intact (online resource 5). Fabrick et al. (2014) reported an insertion of 125 bp at similar position in allele r11 from KMP-6 individual from resistant pink bollworm larvae collected from *Bt* cotton in Khandwa, Madhya Pradesh, India. This supports that larvae which survived in the present study on BGII leaf were resistant one. In the remaining six resistant larvae, as the mutation in cadherin genes are not localized at this particular region, we suggest that the larvae survived on BGII is resistant due to mutation at other regions of the cadherin gene.

This simple leaf disc bioassay method of the present study method can be readily adopted by researchers, for screening or monitoring resistance in PBW and it does not require importation of expensive commercial protein formulations. It may also be useful for evaluating insecticidal activities of new isolates of *B. thuringiensis* or other microbes against PBW. This observation could also be utilized for checking susceptibility of PBW towards new *Bt* cotton transgenic lines.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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