STANDARDIZATION OF LABORATORY AND FIELD METHODS FOR EVALUATING EFFICACY OF TRANSGENIC TECHNOLOGIES.

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Abstract

Assessing the efficacy of transgenic plants under new environmental and management regimes is of prime importance to the companies producing new or improved transgenic products, breeders creating different varieties stacked with Bt endotoxins and growers using them for production. Laboratory and field performance of cotton containing endotoxins should be standardized. Only this can provide accurate and stabilized data for insect control with different transgenic technology. In this presentation, we will also discuss approaches and criteria for mass rearing standardized laboratory colonies of beet armyworm [BAW; *Spodoptera exigua* (Hübner)], fall armyworm [FAW; *S. frugiperda* (J. E. Smith], and bollworm [BW; *Helicoverpa zea* (Boddie)] for laboratory and field evaluation methods for efficacy of Bt cottons.

Introduction

Use of transgenically modified cotton which express an insecticidal protein derived from *Bacillus thuringiensis* Berlinger (Bt) is revolutionizing global agriculture (Head et al. 2005). Cotton is protected from damage of lepidopteran pests. Microbial insecticides are environmentally friendly and highly selective.

In 1996 transgenic cotton, corn Bollgard® (Monsanto Co., St. Louis, MO) encoding the Cry 1Ac insect toxin protein was introduced (Layton 1997). In 2002, Monsanto introduced Bollgard II® (Monsanto Co., St. Louis, MO) which produced the Cry1Ac and Cry2Ab endotoxins (Sherrick et al. 2003). Dow AgroSciences, LLC (Indianapolis, IN) introduced their pyramided-gene technology onto the market in 2004 as Widestrike™ which produced two Bt endotoxins, Cry1Ac and Cry1Fa (Adamczyk and Gore 2004). VipCot is new transgenic cotton in which the active Bt toxin is Vip 3A, an exotoxin produced during vegetative stages of Bt growth (Mascarenhas et al. 2003). Transgenic plants reduce the need for conventional insecticides, providing benefits for human health, and the environment. For example, in U.S. cotton, the average number of insecticide applications used against the tobacco budworm [*Heliothis virescens* (F.)]/bollworm [*Helicoverpa zea* (Boddie)] complex decreased from 5.6 in 1990-1995 to 0.63 in 2005-2009 (Williams 2008-2010).

There are many factors which can affect changes in expressing amount of stacked endotoxins. Individual lepidopteran species vary in their susceptibility to Bt proteins (Luttrell and Mink 1999), and efficacy can be affected by protein expression levels in different plant structures (Adamczyk et al. 2008) and among different varieties (Adamczyk and Gore 2004). Differences in susceptibility can also occur based on the geographic location of the population (Luttrell et al. 1999). Lepidopterans can develop resistance to Bt toxin (Matten and Reynolds 2003; Moar et al. 2008).

Companies and breeders annually produce numerous numbers of transgenic cottons. Because growers use them for cotton production, assessing the efficacy of Bt cotton under new environment and management regimes is of prime importance for all of them. Laboratory and field performance of cotton containing endotoxins should be standardized. Only this can provide accurate and stable data for insect control with different transgenic technology.

The results of the bioassays mortality and damage of beet armyworm (BAW), *Spodoptera exigua* (Hübner), fall armyworm (FAW), bollworm (BW) and cabbage looper (CL), *Trichoplusia ni* (Hübner) on different transgenic cottons are shown, and discussed. Furthermore the criteria needed for developing standardizations of laboratory and field methods can increase and stabilize the evaluation efficacy of transgenic technologies.

Materials and Methods

Field plots

The field trials were conducted in 2005-2009 at the North and South Farms of the Kika de la Garza Subtropical Agricultural Research Center (KSARC ARS-USDA), Lower Rio Grande Valley in Weslaco, Texas. Individual plots were 4-rows (76.2-101.6 cm). Planting date, seeding rate, fertilizer, furrow irrigation, and other production factors were maintained according to local agronomic practices.

Plant material

Plots were planted as recommended for Lower Rio Grande Valley, TX (LRGV) varieties. These included Bollgard®, Bollgard II®, WideStrike™, and non-Bt cottons (Table 1).

Table 1. Cotton Varieties and traits examined

Year	Bt Trait	Variety	Bt endotoxins	Owner of Bt Trait	Owner of Variety
2005, 2006, 2008	Non-Bt	DPL5415 RR	None	None	Delta & Pineland (Monsanto)
2005, 2006, 2008	Bollgard®	NuCOTN 33B	Cry1 AC	Monsanto	Delta & Pineland (Monsanto)
2005, 2006, 2008	Bollgard II®	DPL424 BGII/RR	Cry1 Ac+Cry2 Ab	Monsanto	Delta & Pineland (Monsanto)
2006, 2008	WideStrike™	Phy 485 WRF	Cry1 Ac+Cry2 Fa	Dow AgroScience	Dow AgroScience

Leaves were collected weekly to examine the efficacy of transgenic cotton on lepidopteran larvae, as well as for testing field infestations of cotton plants with different stages of tested lepidopteran for their mortality and damage on Bt and non Bt traits.

Insects

BAW were established from pigweed, *Amaranthus retroflexus* L., BW from cotton, *Gossypium hirsutum* L., CL from cabbage, *Brassica oleracea* L., and FAW from corn, *Zea mays* L. and maintained on a soybean-wheat germ diet (Shaver and Raulston 1971) at the KSARC-ARS-USDA. Insects were obtained from the Vegetable IPM Laboratory Texas AgriLife Research, Weslaco, TX. The use of uniform insects reared on neutral diet helped avoid potentially confounding effects of dietary history on host plant preference. The laboratory strains were established in the mid-1990s from a series of collections around the Weslaco area. Since 2000, the strain has been supplemented with collections of larvae from the same region to retain the vigor of the strain.

Laboratory assays

Leaves were collected from upper and middle of the cotton canopy. Leaf samples were placed into a plastic bag and transported to the laboratory in a cooler with ice. An individual leaf was placed into a Tight-Fit Lid sealing Petri dish (50 x 9 mm, BD Falcon® #351006, VWR International). BAW and FAW were placed 3-5 larvae/dish. Bollworms were placed 1 neonate larval/dish (10 dishes/plot). Five days after exposure in an environmental chamber at 27 ± 1ºC, 65% RH, and a photoperiod of 13:11 (L:D) h larval survival was evaluated (larvae were prodded with a camel-hair brush and considered alive if coordinated movement was observed). Larvae from each dish are scored according to criteria in Table 2. This method was useful for quick assessment.

Table 2. Scoring criteria for bioassays

Condition/Stage	Score	Size insect
Dead	0	0
Alive L1	1	3-5 mm
Alive L2	2	5-8 mm
Alive L3	3	8-12 mm
Alive 4	4	> 12 mm

Surviving larvae after 5 days were given a freshly collected leaf or 7/8 inch leaf disk (or disks for older instars) corresponding to the variety of original treatment and plant age. Survival duration was estimated as lifetime of each larva from the beginning of the experiment until death during the first 15 days after infestation (mean duration of larval stage development on conventional cotton in conditions described above found in Greenberg et al. 2001). Extra collected leaves for this assay were held in the refrigerator.

One-way analysis of variance (ANOVA) tests were used to determine the differences among the survival of the lepidopteran larvae on the different cottons examined (Wilkinson et al. 1992).

Field assays

Artificially infested Bt and non-Bt cottons with egg-masses and larvae of BAW were left under open plots, while BAW pupae and adults were placed on two rows of cotton plants in commercially-produced cages: metal tubes covered with net measuring 1.8 x 1.8 x 1.8 m (BioQuip, Gardena, CA).

1. **Infesting with BAW egg masses**. Egg masses were deposited on waxed paper and placed in adult rearing cages (1 L cardboard ice-cream containers). Egg masses of equal size (ca. 100-150 eggs/3.0 cm^2) were attached by pinning them to the underside of a mature leaf on every second plant. Infestations were conducted at 60 and 90 days after planting (DAP). BAW populations were estimated per plot at 8-10 days after infestation using a 1.2 m drop cloth placed at 3 random locations within the center rows. The plants were shaken to drop the larvae. In addition, the condition of eggs (e.g., desiccation, predation) and the amount of leaf damage was examined.
2. **Infesting with BAW larvae**. This study was initiated in 2006 at the North Farm on five different cotton varieties. Each plot consisted of 2 cotton rows with a total of 90 plants (45 per row). Two infestations of 5-10 neonate larvae per plant were made at 70 DAP using the Davis inoculators, and 80 DAP with 1st and 2nd instars, using a salt shaker. Larvae were mixed with sterile corn cob grits (20/40 mesh) in the supplied plastic inoculator bottle. After seven days, the number of live larvae and damaged leaves were estimated as described above.
3. **Infesting with BAW pupae**. Pupae were released in commercial cages. A total of 180 pupae (50% female) were released in each cage by placing them in a paper cup attached to the top of the cage at 80 DAP. The cotton varieties were evaluated for BAW emergence and leaf damage 10 days after the pupae were placed in the cage.
4. **Infesting with BAW adults**. Adults were released into commercial cages (125 adults, 50% females/cage) at 50 and 90 DAP. In the LRGV adults were released after 8 pm. After 10 days BAW larvae were sampled using drop cloths and leaves were inspected for damage.
5. **Visual observation**. Survival of the different stages of BAW after artificially infesting Bt and non-Bt cottons with BAW in field conditions were recorded, as well as leaf damage by feeding BAW larvae (percentage of leaf damage from total recorded) and rate of damage. Leaf rate of damage was estimated based on the following four categories: 0 - no apparent damage; 1 - light feeding damage or ≤10% leaf area eaten; 2 - moderate damage or 10-30 % leaf area eaten; and 3 - heavy damage or >30.0% leaf area eaten.

Results and Discussion

In all laboratory bioassays, the mortality of neonates was on average 5.8-fold higher when they fed on Bt-cottons compared with non-Bt (t=13.3, P= 0.0001). BAW, CL, BW, and FAW had higher average mortality on dual Bt type varieties (85.4%) than single Bt (45.3%) (t=11.9; P=0.0001). BW with 68.8% average mortality was more susceptible to Bollgard® compared to BAW (35.2%), CL (50.4%), and FAW (49.7%) (P=0.001). Mortality of BAW after feeding on Bollgard II® and WideStrike™ Bt traits was 74.1%, and significantly lower than those on CL (95.5%), BW (90.4%), and FAW (87.2%) (P=0.001) (Figure 1).

For 2006-2009, we did thousands of laboratory bioassays from different Bt trait varieties. Our results showed the same trend of susceptibility of tested lepidopteran to Bt traits as described above. But in the process of investigating, we observed variable data despite the same treatments and the same initial insects.

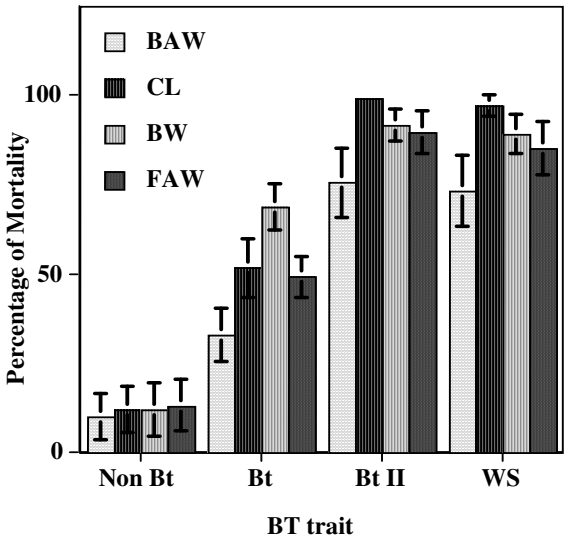

Figure 1. Mortality of lepidopteran neonates on different Bt traits

Laboratory efficacy bioassays of transgenic technologies need to be standardized. At first, a standard requirement should be developed to test insects and transgenic (non-transgenic) plants. The initial insects' survival must be not less than 95%, with an age of neonate larvae of 3 d after hatching (neonate larvae 0 d after hatching are very sensitive to mechanical damage and high mortality when transferred from diet to test leaves). Larval mortality on

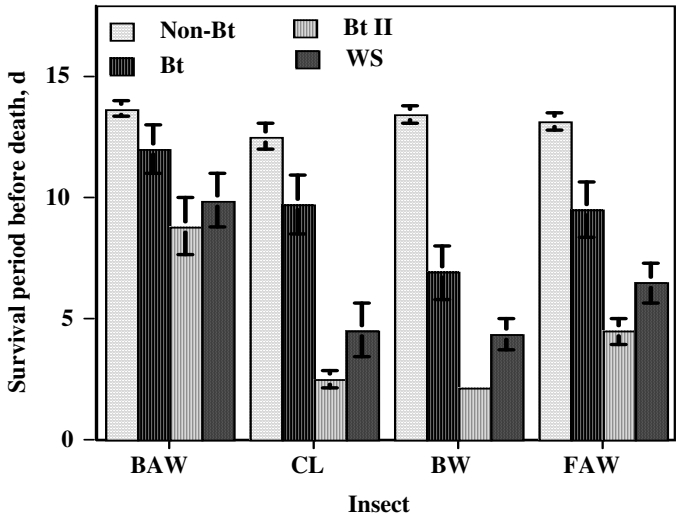

Figure 2. Survival duration of neonates on different Bt traits

transgenic plants can be assessed after plant tissue ingestion and the plants received some level of damage (Hardee et al. 2001). We observed that when the larvae fed on less effective Bt type cotton leaves, they needed to consume more leaf material to reach the level of endotoxins that provide the larvae mortality. BAW needed to feed on Bollgard® an average of 12 d, while BW only 7 d before larvae began to die. BAW needed to consume leaf material on Bollgard II® an average of 9 d, FAW 4 d, CL 3d, and BW 2 d, while on WideStrike™ they needed 10 d, 6 d, 5 d, and 4 d, respectively (Figure 2).

Some larvae will continue to live for two to three days after feeding has stopped. Mortality should be estimated after larvae are found dead, but an express-method of lepidopteran larval survival can be estimated at 5 days after exposing larvae to tested leaves. Larvae from each dish are scored according to criteria in Table 2.

The initial population of tested insects reared on an artificial diet may have caused a reduction of feeding after being transferred to natural host plants. The genetic consequences of mass rearing lepidopteran must be considered prior to testing on cotton with and without Bt traits. The genetic processes that can occur in small isolated cultures during periods of rapid growth are a function of the size of the initial culture and its genetic variability. Thus, the initial culture should be started with a population large enough to ensure enough alleles for heterozygosity for multiple generations. The minimum number of individuals required to initiate an adequate culture and preserve the genetic background is >2,000 individuals of field collected lepidoptera. After the establishment of the initial culture, the main problem is maintaining and increasing the colony under laboratory conditions. The laboratory culture of lepidoptera begins with the F_1 progeny of the insects collected from the field that reproduce to make the F_2 progeny. Rearing the insects on natural host plants for about 2-3 generations will increase the number of insects in culture and help stabilize the colony. This presents such genetic problems such as “bottle necks” and the lack of natural selection pressures in laboratory culture by rearing lepidoptera from initial culture through natural host plants, their qualitative indices are stabilized and are similar to those in “wild” populations. The lepidopteran colony can be maintained in field insectaries or greenhouses, and then transferred to artificial diet for 8-9 generations. Differences in susceptibility can also occur based on geographic location of populations (Luttrell and Mink 1999). Populations of the lepidopteran that feed on different plant species can be classified as genetically differentiated host-associated strains which may differ in their susceptibility to ð-endotoxin present in transgenic crops. Fall armyworms collected from bermudagrass were significantly more susceptible to Bt cotton than larvae collected from field corn. Other agronomically important lepidopterans, such as tobacco budworm, *Heliothis virescens* (F.), cotton BW, and pink bollworm, *Pectinophora gossypiella* (Saunders), differ in their susceptibility to the ð–endotoxin found in the foliar Bt product (MacIntosh et al. 1990) as well as transgenic Bt cotton (Wilson et al. 1992, Halcomb et al. 2000).

Cannibalism may be the most important mortality factor in populations of BW (Stinner et al. 1977). Generally, a decreasing food supply increases the frequency of cannibalism (Fox 1975). Polyphagous herbivores use a large number of food sources, and show a preference for certain plant species over others. Low food quality also increased cannibalism.

Feeding on transgenic cottons significantly reduced pupal weight, emergence, and delayed larval development.

Standardization of the plants used in bioassays need to include the day when plant samples are collected, how many times per day the plants will be collected, the side of the plant, and the amount of endotoxins in different plant structures. The terminal leaf of cotton is preferred for laboratory bioassays of lepidopteran larvae. The quantification of the endotoxins is needed because there are differences in endotoxin levels in the leaves and among the other cotton plant structures. Leaf samples should be placed in a plastic bag and transported to the laboratory in a cooler with ice packages to prevent the loss of the endotoxin levels. Analyses on the leaves should be conducted on fresh leaves, although, if frozen, the endotoxins can be determined for about one year. Leaf collection should run from 40–120 DAP due to the early and late-season reduction observed in endotoxin levels and the efficacy of Bt cotton (Fitt et al. 1998). The toxin level decreases as the crop matures, and is very low or undetectable in squares (Kranthi et al. 2005) and bolls (Greenplate et al. 2000). Leonard et al. (1997) found no significant difference in the mortality of third instar bollworm larvae feeding on Bollgard® squares as compared to conventional cotton. *Helicoverpa armigera* Hübner and BW larval mortality were greater on the leaves (Arshad et al. 2009) than in fruits. This variability in Cry1Ac toxin expression in different parts of the Bt cotton plant causes variability in the survival and development of target pests (Adamczyk and Gore 2004). The cotton fields from where the leaf samples are obtained for laboratory bioassays should not be sprayed with synthetic chemicals a minimum of three days prior to leaf collection.

Field assays of artificial infestation cotton with different stages of BAW.

Infesting with beet armyworm egg masses. The number of eggs that hatched 3-4 days after exposure to leaves of non Bt cotton ranged from 43.0 to 46.1%. Mortality ranged from 53.9 to 57.0% due to heat. Other factors were desiccation (23.8-31.8%) and predators (25.2-30.1%). Larval survival was the highest on non-Bt cotton (43.6 ± 2.0%), followed by single Bt endotoxin cotton (38.3 ± 4.1%). Survival on non-Bt and single Bt endotoxin cottons were not significantly different from each other. Larval survival on dual Bt cottons was 17.3 ± 4.5% and was significantly less than the survival on non-Bt and single Bt cotton (P=0.024). The percentage of leaf damage was significantly higher on non-Bt cotton, followed by single Bt cotton, then dual Bt (48.0 ± 1.4, 33.7 ± 4.0, and 18.3 ± 1.7%, non-Bt, single, and dual Bt cottons, respectively) (P=0.001) (Figure 3). The rate of damage was 1.7 ± 0.2 for non-Bt cotton, 1.5 ± 0.2 for single Bt, and 0.8 ± 0.1 for dual Bt cottons (P=0.001) (Figure 4). The average number of live larvae per one meter on the non-Bt type of cotton was 3.0 ± 0.6. On single Bt cotton it was 2.2 ± 0.7, and on dual Bt cotton it was 0.2 ± 0.1 (P=0.006) (Figure 5). Previously data showed that in the LRGV, eggs can reduce mortality 2.0-fold when egg-masses are distributed after 8 pm (the heat and heat index fell significantly), and a 1.6-fold reduced mortality from predators when substrate for attaching BAW egg-masses was green in color.

Infesting with beet armyworm larvae. There was 100% mortality of neonate larvae and 71% mortality of 1st and 2nd instars 1-2 days after cotton infestation in LRGV due to heat and physical damage. Of the surviving larvae, damage on non-Bt cotton was 15%, 8.3% on single Bt, and 2.5% dual Bt cotton.

Infesting with beet armyworm pupae. These techniques were the least successful at establishing populations. All pupae were consumed by predators (i.e. fire ants).

Infesting with beet armyworm adults. At 15-20 d after exposure, the average leaf damage on non-Bt cotton was 5.1-fold higher than on dual Bt cottons (P=0.001). The damage to non-Bt cotton was only 1.4-fold higher than on single Bt cotton (P=0.2) (Figure 3). The average rate of leaf damage of non-Bt cotton was 8.9-fold higher than of dual Bt cotton (P=0.001), while the rate on non-Bt was 1.5-fold higher compared to single Bt cottons (P=0.1) (Figure 4). Average numbers of live larvae per one meter on non-Bt type cotton was 7.0 ± 1.6, on single Bt was 2.8 ± 0.5, and on dual Bt was 0.09 ± 0.06 (P=0.006) (Figure 5).

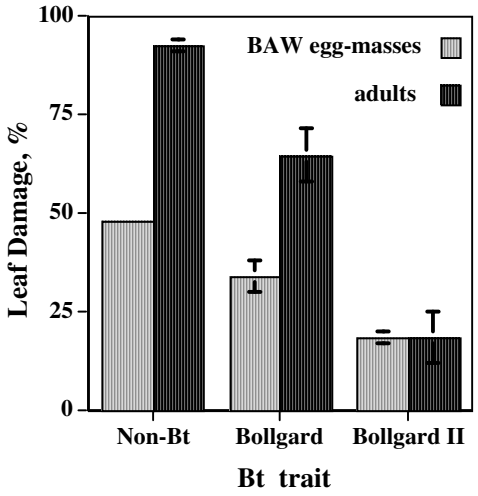

Figure 3. Percent cotton leaf damage.

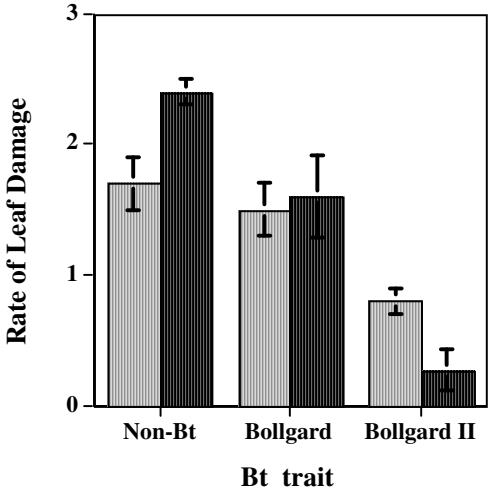

Figure 4. Rate of cotton leaf damage from plants artificially infested with BAW egg-masses and adults (see Figure 3 for legend)

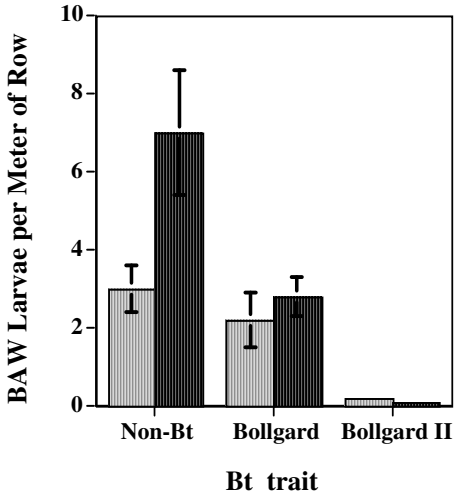

Figure 5. Alive larvae per meter row on cottons artificially infested with BAW egg-masses and adults (see legend from Figure 3)

The artificially infested cotton plants with BAW adults ready to lay eggs were the best method for field assays, while using pupae was the least successful technique for establishing populations of BAW. Artificially infesting with larvae and the rate of infestation with adults and egg-masses need more studies to optimize.

Figure 6. Laboratory assays

Figure 7. Different methods of artificially infesting cotton with BAW in field

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