# DEVELOPMENT OF HYDRATEABLE, COMMERCIALLY-RELEVANT ARTIFICIAL COTTON LEAVES AND ASSAY ARCHITECTURE FOR MONITORING INSECT RESISTANCE TO BT

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## Abstract

Transgenic cotton varieties that express *Bacillus thuringiensis* (Bt) toxins have been adapted readily because they reduce the need of insecticide application, while their environmental impacts are minimal. Recently, pyramided cotton varieties that express two different Bt toxins have been introduced; these varieties may reduce the risk of resistance development in lepidopteran pests populations. However, resistance monitoring in Bt cotton fields are necessary and mandated. Based on a previously described feeding disruption test (FDT) from our laboratory, the objective of this project was to develop a bioassay that uses Bt toxin obtained directly from Bollgard II plant material. The technology under development should be applicable to other pyramided and single gene commercial plants. The assay hardware is a novel 16 well plastic plate with each well of the plate containing a hydrateable, artificial diet containing leaf extract from Bollgard II. Dose-response studies were conducted using a laboratory susceptible strain of the tobacco budworm, *Heliothis virescens*, where different concentrations of leaf extract were used to hydrate a standard meal pad in each well. From these studies, a diagnostic dose was determined of 1.3 µg cotton / meal pad, which essentially stopped the production of fecal pellets by neonates of the budworm after a 24 h incubation. This diagnostic dose was further evaluated using three different populations of *H. virescens* assayed directly from the field from central North Carolina, USA. The diagnostic dose was also evaluated with the Bt resistant, laboratory reared *H. virescens* strains, YHD2 and CxC. The bioassay developed is a practical, reliable monitoring tool that can be used to monitor Bt susceptibility as part of an integrated resistance program in pyramided cotton fields.

## Introduction

The monitoring of insect resistance to transgenic crops that express toxins from the bacteria *Bacillus thuringiensis* (Bt) is not only necessary, but also required by the Environmental Protection Agency (Bates et al., 2005). The area cultivated with Bt cotton has increased gradually in the US since the introduction of these varieties during the 1990´s (May et al. 2003). Bt cotton has several advantages including the decrease of chemical insecticide applications, which benefits the environment and reduces production cost (Sisterson et al., 2004; Shelton et al., 2002). Also, the Bt toxins expressed by the genetically modified cotton plants are highly specific for lepidopterans, but non-toxic to mammals (Roe et al., 1985). However, the major concern of growing Bt cotton –and other Bt crops- remains the loss of susceptibility in lepidopteran pest populations, caused by the continuous exposure to the Bt toxins expressed by the plants (Gould 1998).

To aid in the prevention -or delay- of insect resistance, a new generation of Bt cotton cultivars were developed and became available in the early 2000´s (Jackson et al., 2007). These new cotton cultivars, called "pyramided cotton", express two different Bt toxins that interact with different receptors in the insect gut (Ferré and Van Rie, 2002). Gould (1998) hypothesized that this strategy may lower the probability of resistance development in lepidopteran cotton pest populations, based on the observed low baseline frequency of the resistance allele in the field (Gould et al., 1997). Although models have predicted it is unlikely that lepidopteran pests, such as the tobacco budworm, *Heliothis virescens*, will develop resistance to pyramided cultivars, there are several examples of the plasticity of insects to adapt and evolve resistance to chemical insecticides; thus, it becomes necessary to implement a resistance management program for Bt cotton fields (Shelton et al., 2002). Hence, an integrated resistance strategy based in a monitoring program that uses a reliable and economical monitoring tool is needed.

Bioassays that expose the target pest to Bt toxins could be a useful technique for screening lepidopteran pests in Bt cotton on a large scale (Blanco et al., 2008). Bailey et al. (1998, 2001) developed a practical bioassay based on

larval feeding disruption, to monitor resistance in lepidopteran cotton pests. The Bt toxin Cry 1Ac was incorporated into an artificial heliothine diet, which at the diagnostic dose interrupted feeding of the tobacco budworm and bollworm. In the absence of Bt toxin, neonate larva fed on the artificial diet containing a blue dye, and produced blue fecal pellets that were easily observed on the white background of a novel plate architecture as described by Roe et al. (2000, 2003). Further studies based on the same principle have also estimated a diagnostic dose to Cry 1Ab and Cry 1F toxins for the tobacco budworm and bollworm (Roe et al. 2005). All these previous efforts have used individual Bt toxins mostly expressed in transformed bacteria (Roe et al., 2005). Bt toxins derived from transgenic bacteria are not widely available, are costly to produce and purify, are not produced simultaneously in the same proportions as that found in the cotton plant, are not necessarily the same proteins as that produced by the different commercial plant varieties and are presented to insect in feeding assays devoid of plant material. The use of cotton plants as the source of Bt toxins could represent a more "real world" situation, may be more practical than toxins produced by bacteria and should be more economical. The objective of this study was to develop a reliable, off the shelf larval feeding disruption test using the Bt pyramided cotton variety Bollgard II as the source of Bt toxins, to monitor the susceptibility of field populations of the tobacco budworm.

## Materials and Methods

### Cotton plants and leaf extract preparation

Cotton plants grew under greenhouse conditions, 22-31 ºC and natural daylight throughout the year at the Method Rd. greenhouse facility, North Carolina State University, Raleigh, NC. Seeds were planted in 6-inch clay pots with Fafard 2 growing media (Conrad Fafard, Inc., Agawam, MA) and watered as needed. The cotton varieties planted were: (a) PHY 425 RF (Dow AgroSciences, Indianapolis, IN) and (b) Bollgard II DP 161 B2RF (Monsanto, St. Louis, MO). PHY 425 RF does not express any *Bacillus thuringiensis* (Bt) toxin, while Bollgard II expresses Cry 1Ac and Cry 2Ab Bt toxins.

At 8-weeks after planting, all the leaves of the cotton plants were harvested and stored at -80 ºC until processed. The leaf extracts were prepared by homogenizing leaves from each variety with distilled water, using a proportion of 4-ml of water for each gram of leaf. We used a Polytron homogenizer (PCU, Kinematica, Switzerland), and the resulting mixture was passed through glass wool. The clarified leaf extracts were stored at -80 ºC until used.

### Insects

To determine a diagnostic dose for Bollgard II cotton, we used neonate (0-24 h old) tobacco budworms (*Heliothis virescens*) from the Bt-susceptible strain Hv02, reared under laboratory conditions at the insectary facility of North Carolina State University (NCSU, Raleigh, NC, USA). At the diagnostic dose determined from these studies, two different tobacco budworm strains were used with demonstrated resistance to Bt: YHD2 and CxC. These strains were reared under laboratory conditions in the laboratory of Dr. Fred Gould at NCSU. The strain YHD2 has been selected for resistance to Cry 1Ac, while strain CxC has been selected for resistance to Cry 1Ac and Cry 2A.

### Bioassay description

We based this bioassay on a previous technique described by Roe et al. (2005). Briefly, a 16-well plastic plate was used which has 200 µl of a hydrateable heliothine diet containing a blue indicator dye in each well (the meal pad). To conduct the bioassay, each meal pad was rehydrated with 170 µl of cotton leaf extract from Non-Bt or Bollgard II. The leaf extract was applied to the top of the meal pad and was completely absorbed in approximately 2 h. Afterward, a neonate tobacco budworm larva was placed into each well; to prevent escape, the wells were sealed with a clear plastic sticker, which had small openings to allow the exchange of air. The plates were placed in an incubator (27 ºC, 60% relative humidity, 14:10 L:D) for 24 h. After incubation, the number of blue fecal pellets produced was counted in each well with the aid of a dissecting scope. The blue feces is effortlessly seen on the white background of the plastic well.

### Estimation of a diagnostic dose

A diagnostic dose was estimated for the pyramided cotton variety Bollgard II using the Bt-susceptible strain. A series of doses were evaluated including 0.7, 1.3, 2.6 and 5.3 µg of Bollgard II cotton per meal pad. The leaf extract was diluted with distilled water to obtain the different concentrations, and used to rehydrate the dry meal pads, as described in the previous section. An undiluted Non-Bt cotton treatment was included as a control (42 µg cotton /

meal pad). Four plates, with 16 larvae each were used for each treatment (n=64). The diagnostic dose was defined as the concentration of leaf extract applied to the meal pad that resulted in 0-2 fecal pellets per larvae after 24 h. Leaf mass was expressed as the wet weight of leaf material per meal pad.

**Field evaluation of the bioassay**

To validate that the diagnostic dose was appropriate for the diagnosis of susceptibility in the field, three different tobacco budworm populations were collected from tobacco fields in central North Carolina, USA. The collection sites were (a) Cameron, Moore Co. (08/25/08), (b) Clayton, Johnston Co. (06/30/09) and (c) Reidsville, Rockinham Co. (07/20/09). Eggs attached to tobacco buds were collected in a plastic container and returned the same day to the Dearstyne Entomology Building, NCSU. The eggs were placed in an incubator at 27 ºC, 60% relative humidity, 14:10 L:D until egg hatch. The neonate larvae were then transferred 1 insect per well to meal pads with either Non-Bt or Bollgard II cotton leaf extract at the diagnostic dose (1.3 µg / meal pad), as described previously. The number of fecal pellets was counted after 24 h for each well.

**Evaluation of Bt resistant strains**

We also examined whether Bt resistance could be detected using our diagnostic dose and the same assay approach using YHD2 and CxC neonates under the same conditions described before. The treatments were Bollgard II and Non-Bt cotton at the diagnostic dose of 1.3 µg cotton / meal pad.

## Results and discussion

**Determination of diagnostic dose**

All tobacco budworm larvae that fed on diet treated with Non-Bt cotton produced >2 fecal pellets after 24 h (Table 1). The number of fecal pellets produced per larva varied greatly, from 9 to 79, with an average of 28.5.

Table 1. Estimation of a minimum dose of Bollgard II cotton that produces two or less blue fecal pellets per neonate for the laboratory, Bt susceptible *H. virescens* strain Hv02 (NCSU, Raleigh, NC, USA). (False positive is defined as more than 2 fecal pellets per susceptible neonate at the specified dose tested).

| Cotton variety and dose | Mean no. of fecal pellets at 24 h (± 1SE) | False positives for Bt resistance | Observed range of fecal pellets produced per neonate |
|---|---|---|---|
| Non-Bt | | | |
| 42.5 µg/ meal pad | 28.5 (± 1.3) | | 9 - 79 |
| Bollgard II | | | |
| 0.7 µg/ meal pad | 2.9 (± 0.8) | 20.3% | 0 - 31 |
| 1.3 µg/ meal pad | 0.0 (± 0.03) | 0% | 0 - 1 |
| 2.6 µg/ meal pad | 0.1 (± 0.04) | 0% | 0 - 2 |
| 5.3 µg/ meal pad | 0.3 (± 0.04) | 1.6% | 0 - 4 |

The diagnostic dose for the detection of susceptibility to Bollgard II cotton was defined as the lowest dose tested that produced no more than 2 blue fecal pellets per larva in any of the susceptible neonate tobacco budworm larvae tested. Susceptible, laboratory reared larvae that fed on artificial diet treated with a concentration of 1.3 µg Bollgard II cotton / meal pad produced ≤ 2 fecal pellets after 24 h (Table 1). Thus, the diagnostic dose was 1.3 µg of Bollgard II cotton / meal pad with an error rate for the diagnosis of susceptibility of 0%. This diagnostic dose was used for the studies that follow. An example of wells with Non-Bt and Bollgard II cotton containing a single larva per well after a 24 h incubation is shown in Figure 1.

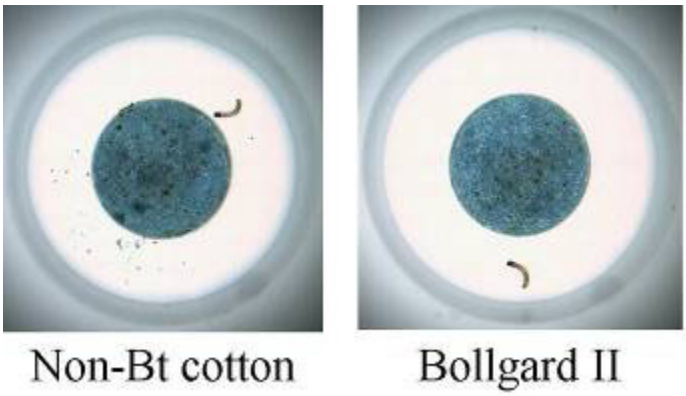


Figure 1. Heliothine diet rehydrated with Non-Bt and Bollgard II cotton leaf extract (1.3 µg/ meal pad). A single tobacco budworm (*H. virescens*) larva was placed in each well and allowed to feed for 24 h; then the number of fecal pellets was counted (Non-Bt cotton, mean = 28.5; Bollgard II cotton, mean = 0 fecal pellets)

**Field evaluation of the diagnostic dose for Bt susceptibility**

Field evaluation of the bioassay was conducted with tobacco budworm samples from Moore, Johnston and Rockinham Co., North Carolina, from collections during 2008 and 2009. Neonate larvae collected as eggs from tobacco were allowed to hatch in the laboratory. Neonates allowed to feed for 24 h on Non-Bt cotton meal pads produced > 2 fecal pellets on average, while larvae exposed to Bollgard II treated diet did not produced any fecal pellets (Table 2).

Table 2. Validation of the Bollgard II cotton diagnostic dose of 1.3 µg cotton / meal pad with neonates of the tobacco budworm, *H. virescens*, collected as eggs from tobacco in central North Carolina.

| Tobacco field site where eggs were collected | Cotton variety | Mean no. of fecal pellets at 24 h (± SE) | False positives for susceptibility |
|---|---|---|---|
| Cameron, Moore Co. | Non-Bt | 10.1 (± 1.4) | -- |
| | Bollgard II | 0 | 0% |
| Clayton, Johnston Co. | Non-Bt | 24.7 (± 2.2) | -- |
| | Bollgard II | 0 | 0% |
| Reidsville, Rockinham Co. | Non-Bt | 13.0 (± 1.5) | -- |
| | Bollgard II | 0 | 0% |

The assumption was made that the tobacco budworm populations in our collection sites are susceptible to Bt, more than a reasonable assumption based on the lack of any reports of significant resistance to Bt in the continental US. Since no blue fecal pellets were produced at the Bollgard II diagnostic dose for any of the insects tested from the field (equal to 0% false positives for Bt susceptibility, Table 2), it was concluded from these studies that the assay was successful in the diagnosis of Bt susceptibility. However, in the Non-Bt treatments, 3.3% of the larvae tested from the Cameron site and 2.2% from Reidsville did not feed. Although this non-feeding rate was observed, the percentage of individuals was minimal. Bailey et al. (2001) made similar observations with field collected larvae

from Louisiana, North Carolina, Georgia and Mississippi. This did not occur for laboratory reared insects (see Non-Bt, Table 1) where the overall fecal production was also higher than that for field collected neonates (see Non-Bt for all three populations, Table 2) and might suggest that laboratory adaption to artificial diet may play a factor in these non-feeding events.

**Evaluation of the diagnostic dose with laboratory strains resistant to Bt**

Assay responses were also examined with 1.3 µg of Bollgard II cotton versus 1.3 µg of Non-Bt cotton for two laboratory strains of the tobacco budworm resistant to Bt. Almost 78% of the neonate larvae from strain YHD2 that fed on Bollgard II treated diet produced > 2 fecal pellets, while 100% of YHD2 larvae that fed on diet treated with Non-Bt cotton produced > 2 fecal pellets (Table 3). Although the assay as a whole was successful in the diagnosis of resistance in the insect population tested, the percent resistance was only 78.1% (Table 3). There are several possible explanations for this outcome. For example, multiple toxins were present in the assay with the insects having different levels of resistance to these toxins. Also, the YHD2 insects no longer can successfully complete their development on cotton because of laboratory selection on Bt treated artificial diet. Another explanation is that the diagnostic dose was too high. Note that there was a significant difference in the number of fecal pellets produced by YHD2 larvae that fed on Non-Bt cotton and Bollgard II cotton treatments ($t= 7.69$, $P< 0.0001$) (Table 3). This difference could also be affected by other plant traits between treatments irrespective of the presence or absence of Bt toxin or further suggest that the diagnostic dose should be reduced.

Table 3. Response of the Bollgard II cotton diagnostic dose of 1.3 µg cotton / meal pad, with the Bt resistant strains YHD2 and CxC strains (NCSU, Raleigh, NC).

| Tobacco budworm strain | Cotton variety | Mean fecal pellets produced at 24 h (± 1SE) | Percent resistance | Observed range of fecal pellets produced per neonate |
|---|---|---|---|---|
| YHD2 | Non-Bt | 30.1 (±2.1) a | -- | 10 - 60 |
| | Bollgard II | 12.4 (±1.9) b | 78.1% | 0 - 74 |
| CxC | Non-Bt | 31.7 (±2.8) a | -- | 7 - 78 |
| | Bollgard II | 13.9 (±1.6) b | 90.3% | 0 - 62 |

Similar results were obtained when the diagnostic dose was evaluated with neonate larvae from the Bt-resistant strain, CxC. More than 90% of the CxC larvae that fed on Bollgard II treated diet produced > 2 fecal pellets, while all larvae that fed on Non-Bt cotton treated meal pads produced > 2 times the number of fecal pellets. This observed difference was also statistically significant ($t=6.49$, $P<0.0001$).

The observed false negatives, that is 21.9% and 9.7% of larvae from strain YHD2 and CxC, respectively, may be due to the level of cross resistance to Bt toxins. The strain YHD2 has been selected for resistance to Cry 1Ac (Jackson et al., 2007), and while some instances of cross resistance to other Bt toxins (Cry 1Ab and Cry 1Fa) have been observed in *H. virescens* (Ferré and Van Rie, 2002), the levels of expression of Cry 1Ac in Bollgard II cotton and the level of cross resistance to Cry 2Ab may explain the susceptibility of 21.9% of the YHD2 larvae. Likewise, the strain CxC was originally selected for resistance to Cry 1Ac, and then for Cry 2A (Jackson et al., 2007). The levels of Bt toxins expression and levels of cross resistance may also explain the 9.7% of false negatives of the CxC larvae. As discussed already, there are other factors that can affect these results. Regardless, the population as a whole was diagnosed as demonstrating resistance to Bt and as shown earlier, the assay was essentially 100% successful in the detection of susceptibility in field populations.

In general, our results show that feeding disruption tests using cotton leaf extracts as the source of Bt toxins to rehydrate an artificial diet can be used to monitor resistance of tobacco budworm populations to Bollgard II cotton. A similar bioassay has also been developed for another pyramided cotton variety, Widestrike, which expresses Cry 1Ac and Cry 1F (data not shown). The advantages of this assay approach for monitoring for Bt toxin susceptibility are the following: (a) an unlimited, easy to obtain supply of the insect toxin is available from plant extracts which can be stored for extended periods by freezing; (b) standard blank, hyrateable meal pads can be used (simplifying

production), charged with any plant derived toxin as needed and then dehydrated before storage at room temperature (studies have been conducted to show that hydrateable cotton meal pads are stable at room temperature for 5 months; data no presented); (c) the assay format is easily adaptable to new insect resistant plant varieties as they come on line (just obtain plant homogenate); (d) the assay is easily applicable to commercially relevant, multiple plant toxins of any type (protein or nucleic acid); (e) the technique produces a ready-to-go, off the shelf diagnostic (just add water and the insect); and the assay is easy to read (look for the presence or absence of a blue indicator on a white background).

## Summary

A bioassay based on a larval feeding disruption test (FDT) was developed for resistance monitoring in neonates of the tobacco budworm, *Heliothis virescens*, to the pyramided cotton variety Bollgard II. We determined a diagnostic dose of the Bollgard II leaf extract of 1.3 µg / meal pad. Susceptible tobacco budworm neonate larvae feeding on this dose produced ≤ 2 fecal pellets in 24 h, while resistant larvae produce > 2 fecal pellets. The diagnostic dose was successful essentially 100% of the time in diagnosing susceptibility to Bt toxin in three field populations of the budworm collected from North Carolina, USA. The assay was also successful as a whole in the detection of resistance in two different laboratory populations of Bt resistant budworms, although there were some false negatives. This bioassay is a practical and reliable monitoring tool to measure Bt susceptibility to pyramided cotton that can be integrated into an overall resistance monitoring program.

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