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EFFICACY OF SINGLE AND DUAL GENE COTTON *GOSSYPIUM HIRSUTUM* EVENTS ON NEONATE AND THIRD INSTAR FALL ARMYWORM, *SPODOPTERA FRUGIPERDA* DEVELOPMENT BASED ON TISSUE AND MERIDIC DIET ASSAYS[1]

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[1]Presented verbally at The Armyworm Symposium held in conjunction with the Entomological Society of America Southeastern Branch Meeting, March 6-10, 2010 in Atlanta

ABSTRACT

We evaluated mortality and developmental parameters of fall armyworms, *Spodoptera frugiperda* (J. E. Smith), to the single *Bacillus thuringiensis (Bt) cotton trait,* Bollgard® and dual Bt cotton traits (Bollgard II® and WideStrike™) by using a cotton leaf-tissue assay and by incorporating lyophilized cotton tissue into a meridic diet. Bioassays were conducted for both neonate and 3rd instars. Leaf tissue bioassays indicated that Bollgard II® and WideStrike™ are highly effective against fall armyworm neonates by causing mortality and by retarding development parameters such as larval weight, pupal duration, and time to adulthood. Bollgard® was not significantly different from non-transgenic cotton in terms of mortality or feeding, with the exception of the non–Bt (PhytoGen 425RF), which had an inherent form of resistance that is not associated with a transgenic event. Third instars evaluated with lyophilized diet bioassays were not as affected by the Bt traits to the same degree as neonates; however, larval weights were lower, and developmental parameters such as time to pupation and time to adulthood were longer. The duration of pupal development was significantly longer for 3rd instars that survived the highest dose of 5,000 µg of WideStrike™ cotton tissue. Sublethal doses for Bollgard II® and WideStrike™ were generally observed at 500 to 5,000 µg of lyophilized cotton tissue per mg of meridic diet, depending upon the variable (time to pupation, pupal duration, time to adult emergence) measured.

Key Words: *Bacillus thuringiensis,* Cry1Ac, Cry2Ab, Cry1F + Cry1Ac, GMO, PIP, transgenic

RESUMEN

Evaluamos los parámetros de mortalidad y desarrollo del gusano cogollero, *Spodoptera frugiperda* (J. E. Smith), hacía una sola cepa algodonera de *Bacillus thuringiensis (Bt),* Bollgard® y una cepa algodoner dobre de Bt (Bollgard II® y WideStrike™) usando tejido de las hojas de algodón e incorporando tejido de algodón liofilizado en una dieta méridica. Se realizaron bioensayos en larvas recién nacidas y del tercer instar. Los bioensayos con tejidos de hojas indican que Bollgard II® y WideStrike™ son muy efectivos contra las larvas recién nacidas de cogollero al causar mortalidad y por demorar los parámetros de desarrollo como el peso de las larvas, la duración de la etapa de la pupa y el tiempo de llegar a la etapa del adulto. Bollgard® no fue significativamente diferente que el algodón no transgénico en terminos de la mortalidad y alimentación, con la excepción de (PhytoGen 425RF) sin Bt, que tenia una forma natural de resistencia que no fue asociada con un evento transgénico. Los instares de tercer estadio que fueron evaluados con bioensayos de dietas liofilizados no fueron afectadas por las características de Bt al mismo grado que las larvas recién nacidas; sin embargo, el peso de las larvas fue menor, y los parámetros de desarrollo como el tiempo de la pupación y el tiempo para llegar al estado del adulto fueron mas largos. La duracióon del desarrollo de las pupas fue significativamente mas larga para los instares de tercer estadio que sobrevivieron la dosis mas alta de 5,000 µG de WideStrike™ del tejido de algodón. Las dosis subletales para Bollgard II® y WideStrike™ fueron observadas generalmente a los 500 to 5,000 µg de tejido de algodon liofilizad por mg de dieta méridica, dependiendo sobre las variables medidas (tiempo de pupación, duración del estadio pupal, tiempo del emergencia del adulto).

Translation provided by the authors.

The fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith), has increased in importance as a pest of cotton, *Gossypium hirsutum* (L.), and other crops in the southern cotton belt over the last 5 years (Leonard et al. 2006). However, the bollworm, *Helicoverpa zea* (Boddie), and the tobacco budworm, *Heliothis virescens* (F.), remain important heliothine pests that cause the

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most damage and yield loss throughout the cotton belt (Williams 2009). Fall armyworms do not diapause or overwinter in temperate climates (Luginbill 1928; Pair et al. 1986; Raulston et al. 1986; Pair et al. 1991), but populations survive the winter in northern Mexico and southern Texas, Florida where they build on corn (*Zea mays* L.). Subsequent generations migrate north as the season progresses (Pair et al. 1991). Because of the transient nature and unpredictable infestations of FAW in cotton, it has been difficult to develop management strategies and tools for control. Insecticides have been used almost exclusively for FAW control in cotton with marginal, or below acceptable results. Both insecticide resistance (Yu et al. 2003) and the inherent disruption of beneficial insects can occur depending upon the insecticide mode of action. Foliar insecticides applied to young larval infestations are much more efficacious as opposed to later instars (Mink & Luttrell 1989). Larger, mature larvae are harder to control with insecticides because of their inherent behavior of being lower in the cotton canopy and because they burrow into cotton fruit (Ali et al. 1990), reducing exposure to insecticides.

More recent technology for control of lepidopterous pests has been the development of transgenic cotton varieties with *Bacillus thuringiensis* Berliner subsp. kurstaki (Bt), Bollgard®, which codes for δ-endotoxin Cry1Ac endotoxin protein; Bollgard II® which encodes δ-endotoxin Cry1Ac + Cry2Ab proteins; and WideStrike™ which encodes δ-endotoxin Cry1F + Cry1Ac endotoxins. Bollgard® cotton does not provide adequate mortality to FAW but does inhibit feeding and larval development (Adamczyk et al. 1998; Stewart et al. 2001). Improvements in mortality and subsequent control were made with the release of Bollgard II® in commercial cotton varieties (Stewart et al. 2001; Chitkowski et al. 2003). However, the newer transgenic cotton lines with WideStrike™ technology are currently being evaluated for FAW control in cotton. Even though the Rio Grande Valley of Texas has been documented as the corridor for FAW migration to the central U.S. (Pair et al. 1991), transgenic cotton varieties with Bt technology have just recently been adopted by producers from the southernmost growing regions in Texas. A survey by Cattaneo (2006) reports that of the 633,792 ha of cotton planted in the Rio Grande Valley in 2006, 2.6% had Bollgard® technology, while 2.4% had Bollgard II® technology. Our goal in this research was to evaluate the efficacy and sublethal effects of available Bt technologies on FAW, including the more recently released WideStrike™. The dual-trait technologies may provide a more ecologically sound option for control of FAW, especially during active boll weevil eradication where natural enemies may be negatively impacted by the repeated applications of ultra low volume malathion.

Materials and Methods

Cotton Leaf Tissue Bioassays

Cotton varieties, FiberMax 800 BGRR (Bollgard®), FiberMax 800 BGIIRR (Bollgard II®), PhytoGen 425RF (non-BT), and PhytoGen 485 WRF (WideStrike™) were planted in 8.5-L pots with Sunshine® potting mix in the greenhouse on 29 Sep 2006. When the plants were 80 d of age, fully expanded cotton leaves from nodes 6-8 from were removed and placed in 15-cm diameter Petri dishes lined with Whatman® (Buffalo, NY) filter paper. A few drops of water were added to each dish to maintain the cotton leaves and humidity.

Fall armyworms used in the assay originated from larvae collected from sudan-sorghum (*Sorghum* spp. hybrid) on the USDA-ARS research farm near Delta Lake, Hidalgo, Co., TX, in Jun 2006 and were determined to be the "corn" host strain by R. Nagoshi, USDA-CMAVE, Gainesville, FL. The larvae were reared to adults in an environmental growth chamber maintained at 28.5°C, 65% RH, and a 14:10 h (L:D) photophase on artificial diet (King & Hartley 1985) and were the F6 generation at the time of these assays.

For each cotton variety, there were 10 replicates of a cotton leaf in a Petri-dish, with 5 neonates placed on each leaf. Survivorship/mortality was determined 4, 7 and 10 d after infestation. On the 10th d of the trial, surviving larvae were weighed. The same procedures were used evaluating 3rd instars, with the exception that only 1 larva was placed in each Petri dish to prevent cannibalism, and 3 replicates of 10 larvae were used for each trait. Cotton leaf tissue was collected again from nodes 6-8 to replace the originals after 4 d because >50% of the leaf tissue was consumed in the control.

Percentage mortality for neonates was analyzed with the GLIMMIX procedure of Mixed model analysis; degrees of freedom were calculated by the Satterthwaite method (SAS, 2003, version 9.2, SAS Institute Inc., Cary, NC), and means were separated with LS MEANS ($\alpha = 0.05$) option. Percentage mortality for 3rd instars fed was analyzed by the PROC FREQ procedure (SAS Institute), with differences in percentage mortality determined by the Chi-square test because of the wide range (0% to 100%) of mortality from the bioassays. The relative frequency of Log10 3rd instar weights were plotted for comparisons.

Meridic Diet Bioassays

Leaf tissue from the same cotton varieties used for the cotton leaf tissue bioassays described above were incorporated into a meridic diet. A minimum of 50 g of whole cotton leaf tissue from node 6-8 of the plant were collected on 29 Oct 2006, placed in paper bags, and stored at -80°C.

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The tissue was then lyophilized (Freezemobile, The Virtis Company Inc. Gardiner, NY) and ground through a 20-mesh screen in a Thomas-Wiley Mill (Thomas Scientific, Swedesboro, NJ) on 6 Dec 2006. On 20 Mar 2007, 3 L of a soy-flour and wheat-germ meridic diet (King & Hartley 1985) were prepared at the USDA-ARS insectary, Weslaco, TX and mixed with lyophilized cotton tissue from each variety of non-Bt cotton (FiberMax 800RR and PhytoGen 425RF) and Bt cotton (FiberMax 800BG (Bollgard®), FiberMax 800BGII® (Bollgard II®), PhytoGen 485WRF (WideStrike™) at dosages of 5, 50, 500, and 5000 µg of tissue for each mg of diet. The powdered lyophilized tissue from the cotton varieties was mixed into warm diet with a high speed blender. Diet cups (15 mL, Anderson Tool and Die, Linden, NJ) were filled with 10 mL of warm diet and allowed to cool before placing a single neonate in each of 30 cups for each of the 4 dosages.

Two separate bioassays were conducted for neonates and 3rd instars placed in the diet cups with paper lids. A single neonate was placed in a single diet cup containing 0, 5, 50, 500, and 5000 µg of tissue for 30 replicates of each treatment. Mortality was evaluated at 4, 7, 10, and 14 d from the time larvae were placed on the diet. The larvae were then observed every 24 h for pupation. Pupae were weighed and placed in individual diet cups and the day of adult emergence was recorded. The same procedures were followed for 3rd instars with the exception that larvae were weighed 10 d from the time of being placed on diet. Mortality data were analyzed by the PROC FREQ procedure (SAS 2003) and compared with the Chi-square Likelihood ratio test ($\alpha = 0.05$). Larval weights, time to pupation, and time to adult emergence in days were transformed to the log Poisson distribution because of skewness. Following transformation, the data were analyzed by GLIMMIX mixed model analysis with residuals estimated by the PL method for fitting the data to the linear model. Cotton trait and dose of lyophilized tissue means were estimated with the LS means statement and adjusted and separated by Tukey's ($\alpha = 0.05$) test for determining significance. Significant interactions for cotton trait and dosage of lyophilized tissue were further examined with the SLICE option of the LS MEANS statement in which cotton trait by dosage sliced by cotton trait and the cotton trait by dosage sliced by dosage were examined.

Results

Cotton Leaf Tissue Bioassays

Larval mortality of FAW neonates evaluated on cotton leaf tissue was significantly different at 4 ($F = 24.2$; $df = 4, 45$; $P < 0.001$); 7 ($F = 25.5$; $df = 4, 45$; $P < 0.001$) and 10 d ($F = 28.6$; $df = 4, 45$; $P < 0.001$) (Fig. 1). Bollgard II® and WideStrike® caused near 80% mortality at 4 d and increased to >95% mortality by 10 d. The Bollgard® trait was less effective in killing neonates, and was not significantly different from both non-Bt cottons at 4, 7, and 10 d.

Third instars were highly susceptible to WideStrike® where 100% mortality occurred by 7 and 10 d (Fig. 2). Bollgard II® was responsible for 35% mortality at 7 d and 50% mortality by 10 d, and this mortality was significantly higher than 10 d mortality for larvae reared on non-Bt cotton (FM 800RR).

The relative frequency (log10) of 3rd instars weighed at 10 d showed a distinct distribution of weights for the survivors (Fig. 3). The weights of larvae reared on Bollgard II® were significantly lower and skewed to the left (Fig. 3). The heavier weights of those reared on Bollgard® were more centrally distributed, and non-Bt weights were skewed to the right, averaging 160 mg more than the Bollgard II®, and 120 mg more than the Bollgard® trait respectively. This is an indication that leaf consumption and feeding on Bollgard II® may occur, but the toxins reduce growth and development.

Meridic Diet Bioassays

Mortality for neonate FAW placed on meridic diet was significantly higher for WideStrike™ with mean mortality of 25% for the 500 µg of tissue (Likelihood ratio Chi-square = 39.73; $df = 4$; $P < 0.0001$) and 47% for the 5000 µg of tissue incorporated into diet (Likelihood ratio Chi-square = 145.53.73; $df = 4$; $P < 0.0001$). Mortality for all other entries, including Bollgard II®, was less than 11%. Larval weights for FAW neonates measured after 14 d of feeding were significantly affected by cotton trait ($F = 25.89$; $df = 4, 285$; $P < 0.0001$), dosage of lyophilized tissue ($F = 10.56$; $df = 3, 285$; $P < 0.0001$) and the interaction of cotton trait by dosage ($F = 8.42$; $df = 12, 285$; $P < 0.0001$) for surviving larvae (Fig. 4). At the lower dosages of 5 and 50 µg, feeding on the non-Bt PHY 425RF suppressed weights of larvae resulting in no significant differences from the Bollgard II® or WideStrike™ traits. At 500 and 5000 µg of incorporated diet, larvae feeding on Bollgard II and WideStrike were significantly smaller ($F = 18.32$; $df = 2, 285$; $P < 0.0001$) than those feeding on non-Bt (PhytoGen 425RF). The Bollgard® trait did not significantly reduce larval weights for those at 5 to 500 µg; however at 5000 µg, weights were significantly lower ($F = 12.32$; $df = 2, 285$; $P < 0.0001$) than the non-Bt cottons, but significantly higher ($F = 9.32$; $df = 2, 285$; $P < 0.001$) than the weights for the Bollgard II® or WideStrike™ traits. The non-BT PhytoGen 425RF appears to have some inherent form

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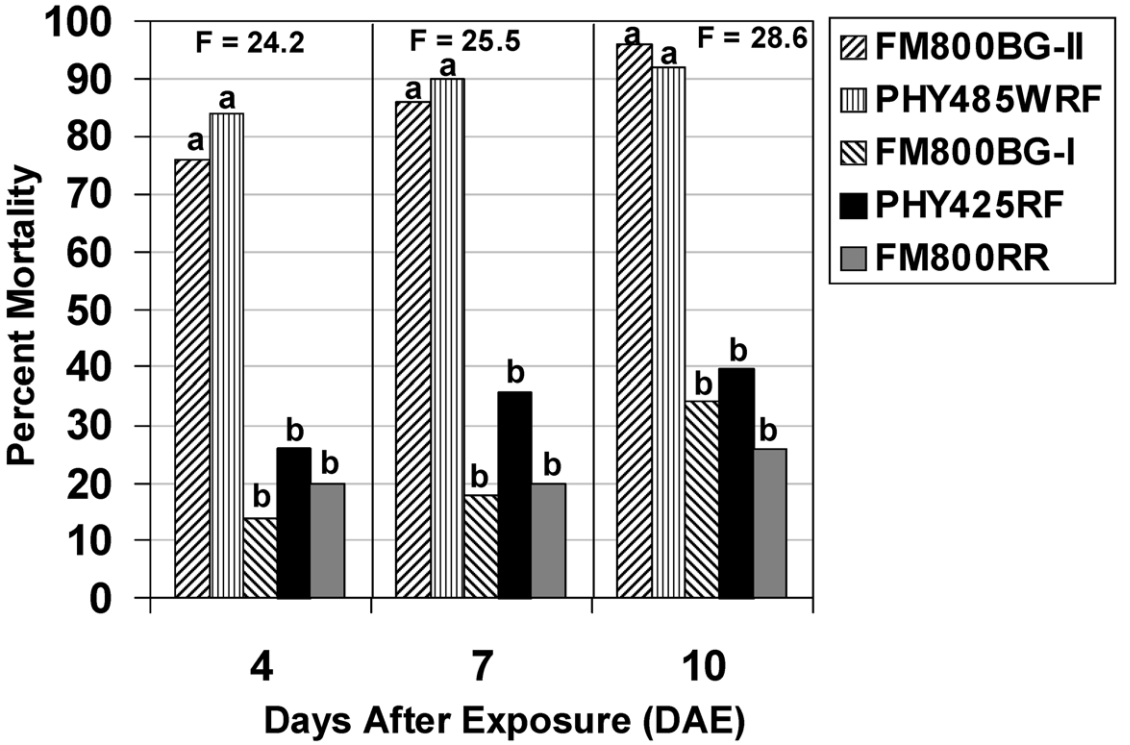


Fig. 1. Larval mortality of fall armyworms placed in Petri dishes as neonates and evaluated for mortality after feeding on Bollgard® (FM 800BGRR), Bollgard II® (FM 800BGII®), WideStrike™ (PHY 485WRF), non-Bt PHY 425RF and non-Bt FM 800RR cotton leaves removed from nodes 6-8.

of resistance that has not been identified, but the detrimental effects of the endotoxins of Bollgard II® and WideStrike™ surpass whatever the effects of the unknown resistance are at the 500 and 5000 µg dosages. The test effects for cotton trait by dosage, sliced by dosage, were significant for 5 µg (F = 3.20; df = 4, 285; P = 0.0137), 50 µg (F = 8.52; df = 4, 285; P < 0.0001), 500 µg (F = 12.05; df = 4, 285; P < 0.0001) and 5,000 µg (F = 25.78; df = 4, 285; P < 0.0001) as were the slice effects of cotton trait by dose, sliced by trait for Bollgard® (F = 3.05; df = 3, 285; P = 0.0288); Bollgard II®, (F = 21.62; df = 3, 285; P < 0.0001); PHY 425RF, (F = 3.71; df = 3, 285; P = 0.0121); and WideStrike™ (F = 21.62; df = 3, 285; P < 0.0001), but what we considered one of the non-Bt controls (FM 800RR) in this study was not significant (F = 0.45; df = 3, 285; P = 0.726) when cotton trait by dose was sliced by cotton trait, indicating that dosage of lyophilized tissue containing not Bt trait had no affect on larval growth and development based on larval weights.

There was a significant response in pupal duration for the neonates based on trait (F = 9.34; df = 4, 285; P < 0.0001), dosage of trait (F = 2.82; df = 3, 285; P = 0.0394) and for the cotton by dosage interaction (F = 2.88; df = 12, 285; P = 0.0009). The trend in significance was that with increasing dosage of trait, especially the Bollgard II® and WideStrike™. Mean development time for pupae increased from 5 to 8 d when compared to the non Bt FM 800RR and PhytoGen 425RF, respectively, (Fig. 5). The test effects for cotton variety by dose, sliced by dose for pupal duration were not significant for any of the cotton varieties at 5 µg, but all other doses (50 µg, F = 4.56, df = 4, 285, P = 0.0106; 500 µg, F = 4.56; df = 4, 285; P = 0.0014; and 5000 µg, F = 8.94; df = 4, 285; P < 0.0001) extended time to pupation (Fig. 5).

The time in days for neonates to develop to adulthood followed a similar pattern as time in pupation (Fig. 6); however, only the main effect of cotton variety (F = 2.57; df = 4, 130; P = 0.0410) was statistically significant, whereas dosage (df = 3, 130; F = 0.44; P = 0.7271) and cotton by dosage (F = 0.91; df = 12, 130; P = 0.5364) were not. The main effects for the 10-d weights of 3rd instars reared on meridic diet were significant for trait (F = 5.30; df = 4, 162; P = 0.0005); dosage of tissue incorporated into diet (F = 4.89; df = 3,162; P = 0.0028) and the

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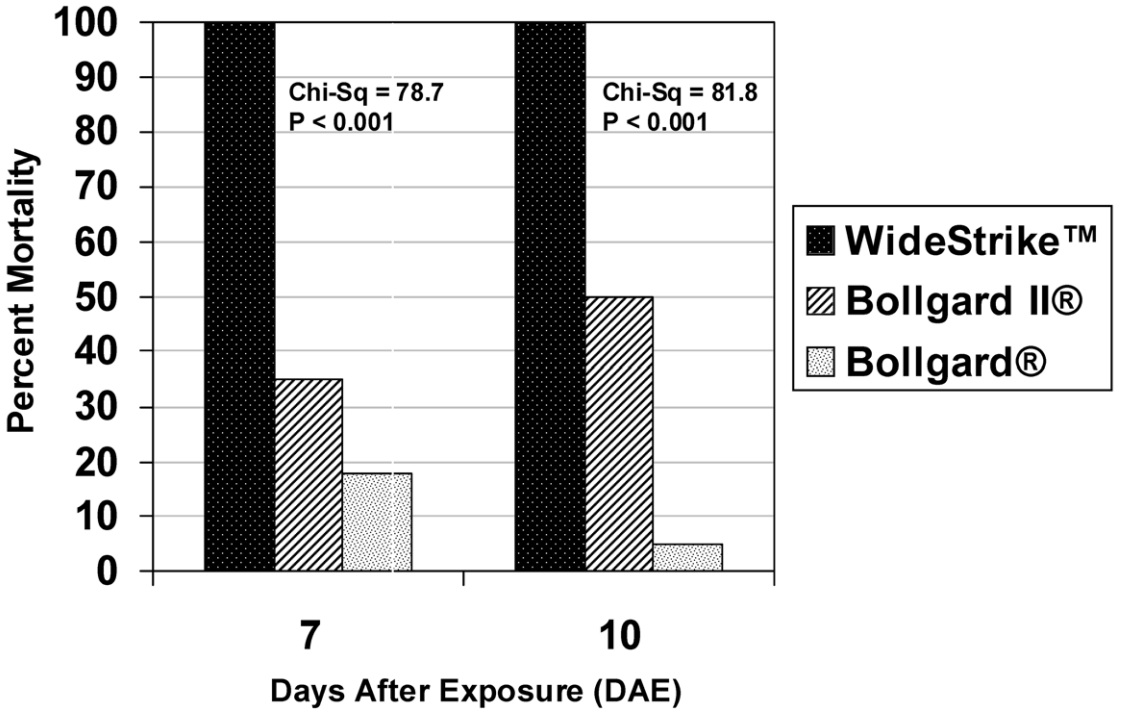


Fig. 2. Larval mortality of 3rd instar fall armyworms at 7 and 10 d after feeding on Bollgard II® (FM 800BGRR), Bollgard II® (FM 800BGII®), and WideStrike™ (PhytoGen 485WRF) cotton leaves removed from nodes 6-8. The non-Bt variety FM 800RR is not shown, and mortality was ≤5%.

cotton trait by dose (F = 2.82; df = 12,162; P = 0.0015) interaction (Fig. 7). Interestingly, the slice effects of cotton by dose, sliced by dose, were only significant for 5000 µg (F = 10.01; df = 4, 162; P < 0.0001), but the cotton trait by dose, sliced by dose was significant for Bollgard II (FM 800BG®II) (F = 4.36; df = 3, 162; P = 0.0055) and WideStrike (PHY 485WRF®) (F = 8.16; df = 3, 162; P < 0.0001). Third instars developed well on the non-Bt (PHY 425RF) incorporated diet based on larval weights, which were significantly higher than all other varieties at the dosages of 500 and 5000 µg, especially when compared to the neonates (Fig. 4). The undetermined form of inherent resistance exhibited in PHY 425RF for neonates does not appear to affect the older larvae.

There were so few 3rd instars that succumbed in the meridic diet study that no data analyses could be conducted. There was no mortality across all treatments at d 4, a total of 4 dead (1.9%) on d 7, and 16 dead (7.6%) after 10 d. The models for the main effects of cotton trait, dosage, and their interactions were not significant for time to pupation, pupal weights, and time to adult emergence for 3rd instars reared on the meridic diet containing lyophilized cotton tissue (Fig. 8). The main effects of cotton trait was significant (F = 3.21; df = 4; 102; P = 0.0159) for the number of days required for pupal development. Fall armyworm reared on the WideStrike™ took on average 5 d longer across all doses when compared to all other cotton types. However the dose or the trait by dose interaction was not significant.

Discussion

Fall armyworm infestations are sporadic, unpredictable, and hard to control in cotton, even with improvements and changes in the expression *Bacillus thuringiensis* endotoxins. It is apparent that one of the more significant improvements in controlling FAW comes from the development of the combination Cry1Ab and Cry1F expression in cotton varieties. In these assays, WideStrike™ provided the most significant effects on FAW mortality and development in terms of larval weights, development times, pupal weights, and successful development to adulthood.

Although we did not quantify the Cry proteins used in the leaf tissue and meridic diet assays,

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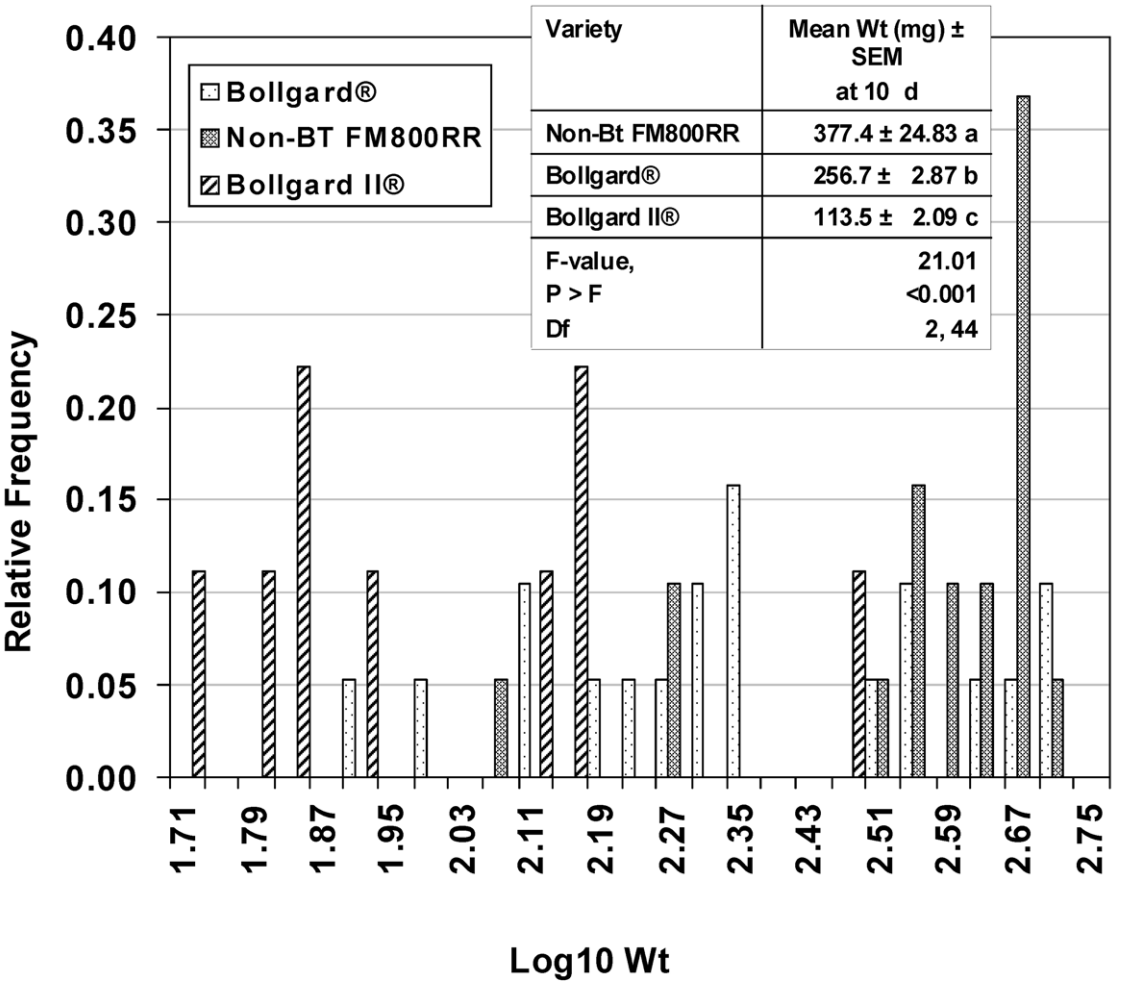


Fig. 3. Relative frequencies and mean live-weights of 3rd instar fall armyworm survivors after 10 d of feeding on non-Bt FM 800RR, Bollgard® (FM 800BG), and Bollgard II® (FM 800BG II) cotton leaves. There were no survivors in the WideStrike™ (PHY 485WRF).

the results give us some summary observations that are of interest when comparing fresh tissue assays with lyophilized tissue incorporated into meridic diet. Fresh cotton tissue from the middle nodes containing tissue from the Bollgard® II and Widestrike® traits can provide close to or above 80% mortality to neonates following 4 d of exposure to leaf-tissue, and near 100% mortality at 7 and 10 d days after exposure. However, when 3rd instars were used in the assays, the WideStrike™ Cry1F combined with the Cry2Ab provides a lethal combination from the fresh leaf tissue, and significantly separates itself from all other technology by providing 100% mortality 7 and 10 d after exposure. Other studies specific to the single and dual traits for Bt for FAW control have reported similar results with middle to upper leaf tissue (Siebert et al. 2008; Adamczyk et al. 2008); however, it has been more recently established that Cry1F is expressed in higher amounts (>85 ppm) collected from the 6th week of flowering from mature leaf tissue collected from the 8th node (Siebert et al. 2009). In addition, the levels of Cry1F were generally higher in all parts of the cotton plant with the exception of flowers. The fact that Cry1F expression increases in older leaf tissue could be advantageous from the standpoint that egg masses are oviposited on the leaves and neonates feed on these leaves before moving to the fruiting structures (Ali et al. 1990).

By comparison, the meridic diet assays as a general rule approached a lethal status for neonates between 500 to 5000 µg of leaf tissue in µg/mL of diet for Bollgard II® and Widestrike in

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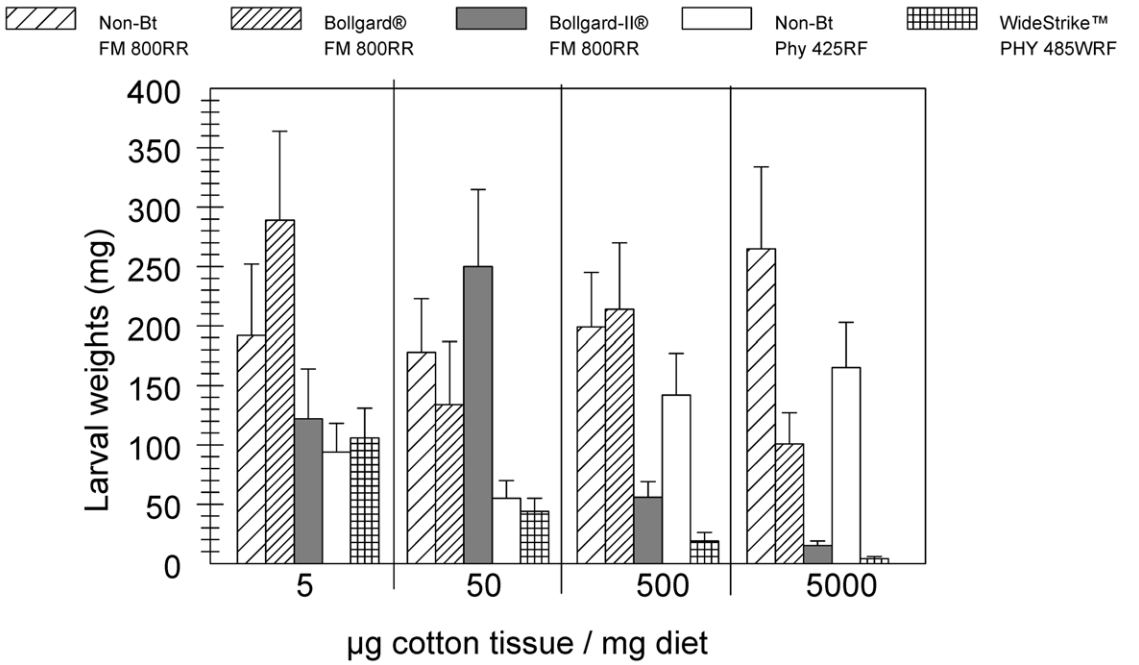


Fig. 4. Larval weights of fall armyworms after being reared from neonates on meridic diet incorporated with lyophilized cotton tissue for 14 d.

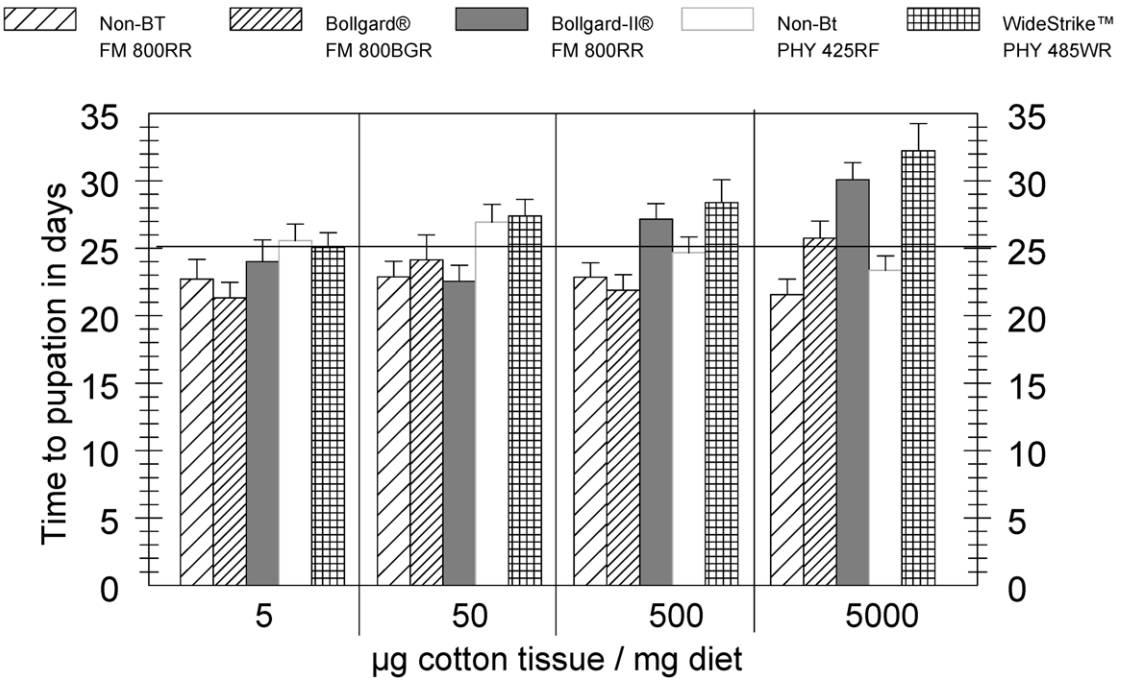


Fig. 5. Time in days to pupation for fall armyworm larvae reared from neonates on lyophilized cotton tissue incorporated into meridic diet for non-Bt FM 800RR, Bollgard® (FM 800BGRR), Bollgard-II®FM 800BGII, non-Bt PHY 425RF, and WideStrike™ (PHY 485WRF). The horizontal line across all doses and varieties represents the overall mean in pupal duration (d) for the assay.

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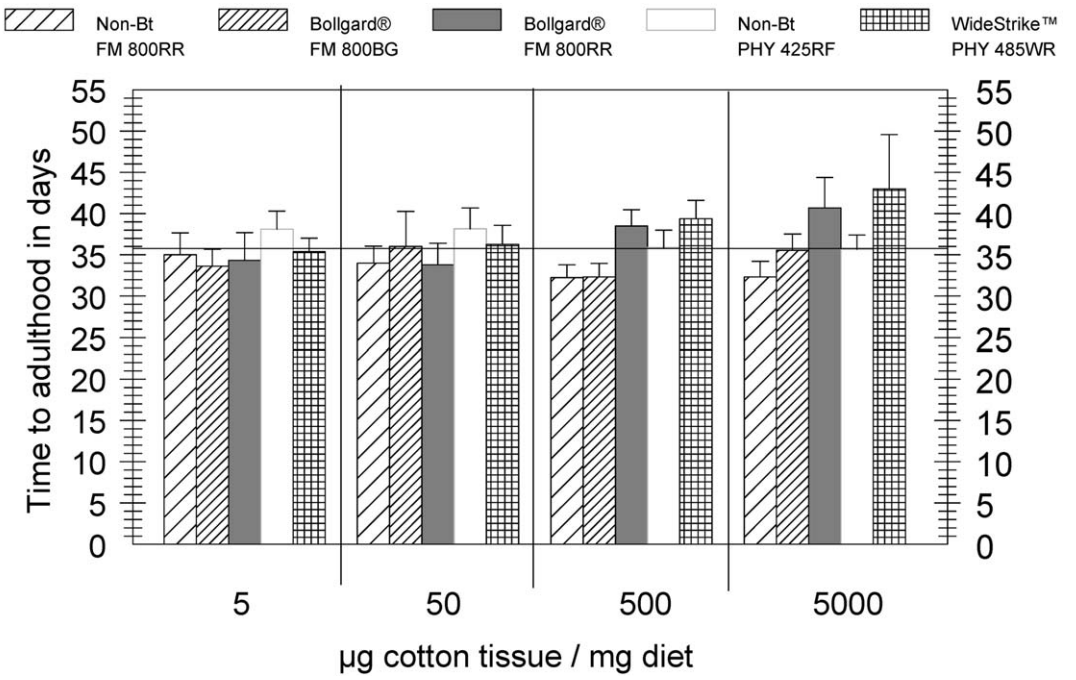


Fig. 6. Time in days to adulthood for fall armyworm larvae that were reared from neonates on lyophilized cotton tissue incorporated into meridic diet for non-Bt FM 800RR, Bollgard® (FM 800 BGRR), Bollgard-II® FM 800BGII, non-Bt PhytoGen 425RF, and WideStrike™ (PHY 485WRF).

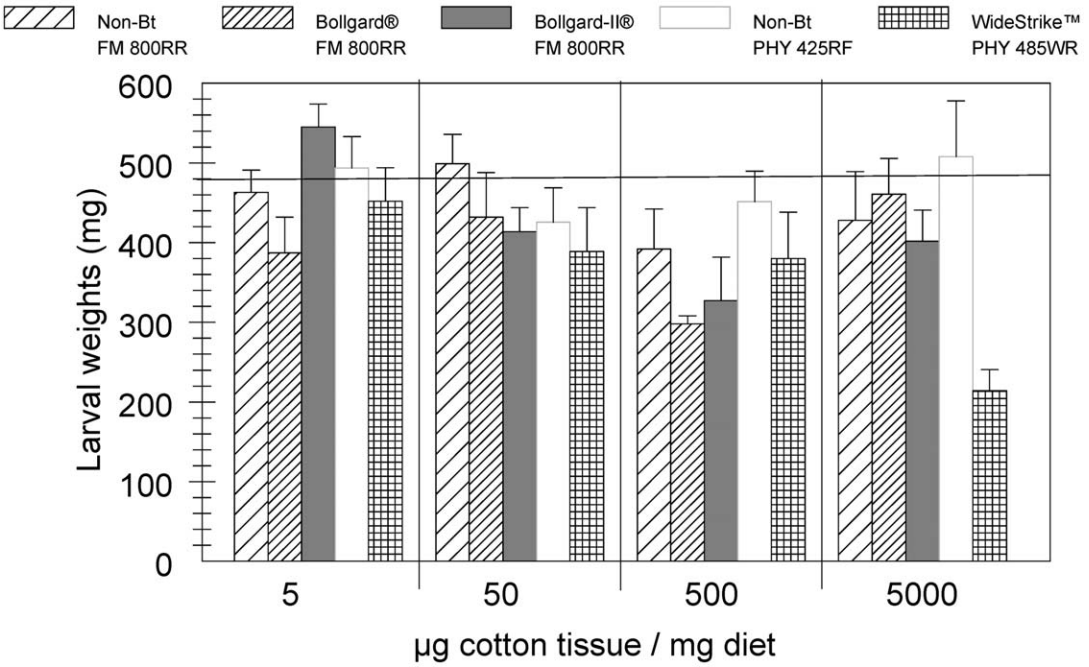


Fig. 7. Fall armyworm larval weights following 10 d of being reared on meridic diet incorporated with lyophilized cotton tissue containing non-Bt FM 800RR, Bollgard® (FM 800 BGRR), Bollgard-II® FM 800BGII, non-Bt PhytoGen 425RF, and WideStrike™ (PHY 485WRF) with the overall mean represented by the horizontal dashed line. Larvae were reared to 3rd instar before being placed on diet.

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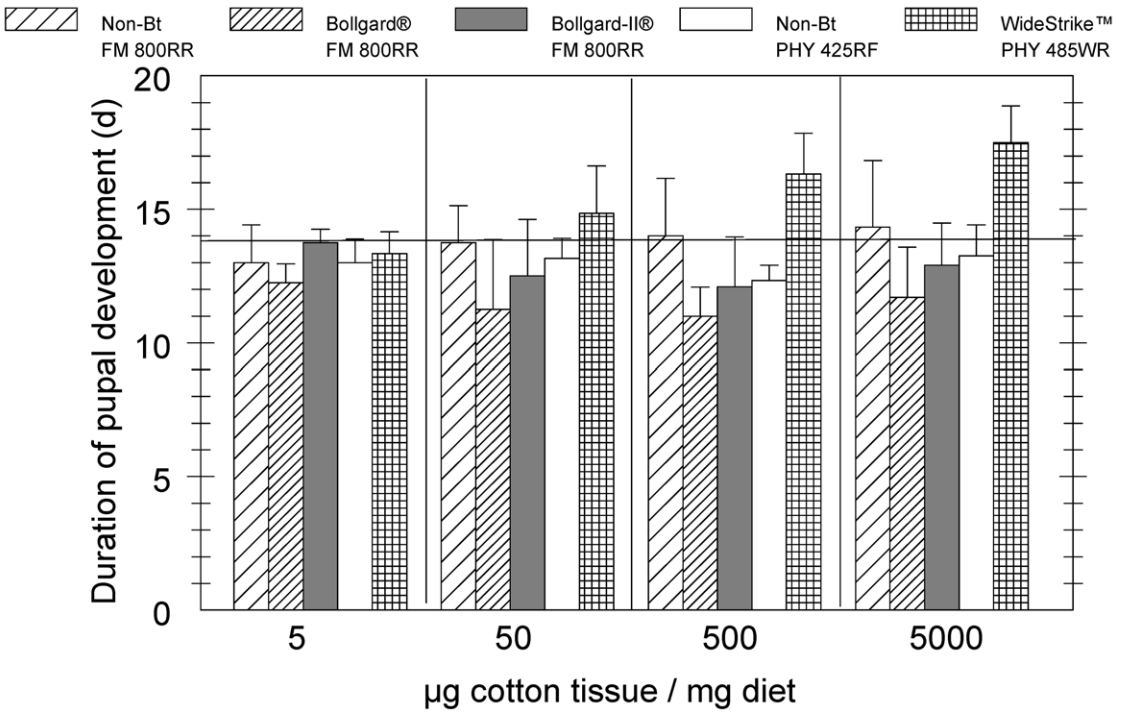


Fig. 8. Fall armyworm pupal duration in days for 3rd instar reared on meridic diet incorporated with lyophilized cotton tissue containing non-Bt FM 800RR, Bollgard® (FM 800BGRR), Bollgard-II® FM 800BGII, non-Bt PhytoGen 425RF, and WideStrike™ (PHY 485WRF) with the overall mean represented by the horizontal dashed line.

terms of larval weights, duration to pupation in d, and time in d to adulthood for the neonates. The effects of the traits and dosages of traits on FAW reared to 3rd instar before exposing them on the meridic diet was much less apparent than that of the neonates. Older larvae have been noted to be harder to kill with different Bt traits (Stewart et al. 2001) and this is good information to have, but infestations will more than likely originate from adults moving into the field, where young larvae feeding on leaf tissue should be exposed to older or younger cotton tissue depending upon the time of infestation.

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