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Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae) Larval Distribution on Different Bt Technologies and Evaluating Cotton Plant Tissue Assays for Resistance Monitoring

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To the Graduate Council:

I am submitting herewith a thesis written by Dawson David Kerns entitled "Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae) Larval Distribution on Different Bt Technologies and Evaluating Cotton Plant Tissue Assays for Resistance Monitoring." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

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Abstract

Field trials were conducted in 2018 and 2019 to determine if an insecticide treatment and different Bt (*Bacillus thuringiensis*) cotton technologies had an effect on bollworm, *Helicoverpa zea* (Lepidoptera: Noctuidae), distribution in the cotton canopy. Non-Bt, Cry1Ac + Cry1F, and Cry1Ac + Cry2Ab cotton varieties were either treated with an insecticide or left untreated after a bollworm, infestation was detected. Cotton plants were mapped for signs of bollworm feeding on floral structures (i.e., bolls, squares, flowers) and the physical presence of larvae. No major differences in the pattern of feeding injury and distribution of larvae were found among the different cotton varieties. Most larvae and damage were found in the middle portion of the canopy. *H. zea* feeding appeared to occur slightly lower in the canopy of cotton treated with a pyrethroid when compared with untreated cotton. Results suggest that a standardized scouting methodology for *H. zea* infestations in cotton could be developed, regardless of if or what Bt technologies were used. Floral structures from the middle portion of the canopy appeared most indicative of *H. zea* infestation levels.

Laboratory experiments were done to evaluate Bt resistance monitoring techniques using purified proteins or various lyophilized cotton plant tissues. Leaves, bolls, squares, white flowers, and pink flowers were collected from non-Bt cotton or cotton varieties expressing Cry1Ac + Cry2Ab, or Cry1Ac + Cry1F + Vip3A. Collected plant structures were lyophilized and ground into fine powders. Diet-overlay assays using purified proteins (Cry1Ac, Cry2Aa, and Vip3Aa39) and cotton plant tissues were conducted on a Bt-susceptible strain and a Cry1Ac, Cry1F, and Cry2Ab-resistant strain

of *H. zea*. The resistant strain was over 95-fold and 54-fold less sensitive to Cry1Ac and Cry2Aa, respectively, compared with the susceptible strain. However, the resistant strain was at least 5-fold more susceptible to Vip3Aa39 than the susceptible strain. Lyophilized boll and leaf tissue from non-Bt cotton severely stunted larval growth, suggesting that these tissues may not be ideal for assessing bollworm Bt resistance. Lyophilized plant tissue from white flowers was best able to detect the differences in susceptibility between the susceptible and resistant strain of *H. zea*.

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Introduction

United States Cotton Production

Upland cotton, *Gossypium hirsutum* (L.), is a major commodity in the United States. In recent years, cotton production in the United States has increased, with nearly 4 million hectares of cotton planted in 2016 and over 5 million hectares planted annually in 2017 through 2019 (USDA-NASS 2020), with 2019 U.S. cotton production being valued at over $6 billion (USDA-NASS 2020). Cotton can serve as a host to a diverse range of insect species belonging to several taxonomic orders. Historically, the Midsouth region of cotton production (i.e., Louisiana, Arkansas, Mississippi, West Tennessee, and the Missouri Bootheel) has experienced higher insect related yield loss and greater insecticide use than the other cotton producing regions of the United States (Luttrell 1994). This heavy insect pest pressure highlights the importance of integrated pest management (IPM) strategies in the Midsouth to reduce both the amount of insecticide inputs and manage insecticide resistance to ensure efficient management of crop pests. These strategies consist of a combination of various management methods that are implemented in a way that accounts for the biological characteristics of pests and their interactions with the environment.

Bollworm

Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae), commonly referred to as the corn earworm or bollworm, is a polyphagous and multivoltine species that has been observed to feed on a wide variety of hosts including corn and cotton (Jackson et al.

2008, King and Coleman 1989). Female moths show a preference for oviposition on host plants during the flowering stage of development (Johnson et al. 1975) and exhibit a high fecundity with an estimated oviposition of 1,000-1,500 eggs over an eight to ten day reproductive period (Fitt 1989). During the fall, larvae pupate in the soil and overwinter until the spring (Stadelbacher and Pfrimmer 1972), at least in the southern U.S., and moths can migrate long distances (Westbrook et al. 1995). Thus, infestations may occur from either local or migrant populations depending on geographic location (Swenson et al. 2013).

Larvae of *H. zea* are a major pest of cotton, preferring to feed on floral structures (i.e., squares, flowers, bolls) (Farrar and Bradley 1985). A single bollworm is capable of feeding on a total of 17 floral structures during its lifetime (Wilson and Gutierrez 1980), thus this species can cause yield losses even at relatively low populations (Adkisson et al. 1964). However, despite being a major cotton pest, *H. zea* tends to exhibit a preference for oviposition on silking corn, *Zea mays* L. (Johnson et al. 1975). As a result, bollworm infestations in cotton often emanate from earlier generations in corn (Lincoln and Isely 1947).

Multiple control methods have been used to reduce yield loss caused by *H. zea*. Historically, pyrethroids had been the primary insecticide applied for the management of bollworm, yet the development of widespread pyrethroid resistance across the United States (Abdelghafar et al. 1993, Brown et al. 1998, Jacobson et al. 2009, Musser et al. 2017), has rendered them less effective. Diamide insecticides (i.e., chlorantraniliprole) are currently recommended for management of *H. zea*, and no meaningful levels of

resistance have yet to be detected in the midsouthern United States (Adams et al. 2016).

Bt Insecticidal Proteins and Transgenic Cotton

Transgenic cotton varieties expressing insecticidal proteins derived from the bacterium, *Bacillus thuringiensis* (Bt), are one of the primary tools used to manage *H. zea*. Cry proteins are delta-endotoxins that constitute parasporal crystals produced by *Bacillus thuringiensis* during sporulation (Bravo et al. 2007). Adang et al. (2014) extensively reviewed the structure and intoxication process of the Cry toxins. Parasporal crystals are solubilized via the cleavage of interchange disulfide bonds when they are ingested by a target insect, thus releasing the protoxin forms. Subsequently, the solubilized protoxins are processed by host gut proteases resulting in active toxins (Adang et al. 2014). Of course, in transgenic plants, the Bt toxins or protoxins are expressed directly as pre-solubilized proteins (Jurat-Fuentes and Crickmore 2017).

A common characteristic among the majority of the Cry toxins is their three-dimensional structure consisting of three distinct domains (Pardo-Lopez et al. 2013). These domains are involved in interactions with midgut proteins (domains II and III) and cell membrane insertion (domain I) (Adang et al. 2014), thus they contribute to the specificity of the toxin (Jurat-Fuentes and Crickmore 2017). Following activation, Cry toxins must pass through the peritrophic matrix before binding to midgut proteins can occur (Rees et al. 2009). Aminopeptidases (APNs), cadherin proteins, alkaline phosphatases (ALPs), and ABC transporter proteins have all been described as Cry-binding midgut proteins associated with mode of action (Adang et al. 2014).

Several different models have been proposed describing the mode of action of Cry toxins, thus the details of the contributing mechanisms associated with target cell toxicity remains controversial (Vachon et al. 2012). The sequential binding model proposes that Cry toxins favor high-affinity binding to cadherin (Pardo-Lopez et al. 2013). It is believed that the binding of the Cry toxin to cadherin allows for further proteolytic processing of the toxin resulting in the subsequent formation of a toxin oligomer with a high affinity for APN or ALP binding (Gomez et al. 2002, Bravo et al. 2004, Pigott and Ellar 2007). It has been proposed that this oligomer may form following the insertion of a monomer into the cell membrane or prior to binding with APN and ALP proteins (Vachon et al. 2012). Binding to APN and ALP proteins occurs in regions of the cell membrane known as lipid rafts (Zhuang et al. 2002) and results in the formation of pores, cell death, and eventual death of the insect due to septicemia (Adang et al. 2014). Alternatively, Zhang et al. (2008) proposed a model that suggests the activation of an oncotic cell death pathway rather than cell membrane insertion as responsible for target cell killing. Additionally, Pigott and Ellar (2007) describe a speculative model that considers both cell membrane insertion and oncotic cell death pathways (Jurat-Fuentes and Adang 2006).

Bt Pyramids

The first Bt cotton expressing Cry1Ac (Bollgard®, Monsanto Co.), was made commercially available in the United States in 1996. Following this release, *H. zea* became a more prominent pest of cotton due to its higher tolerance of Cry1Ac compared with the tobacco budworm, *Chloridea virescens* (F.) (Noctuidae) (MacIntosh et al. 1990, Luttrell and Jackson 2012). Thus, supplemental insecticides were often

needed to maintain adequate control of bollworm (Burd et al. 1999). In 2003, Bt cotton expressing both Cry1Ac and Cry2Ab (Bollgard II®, Monsanto Co.) was commercially released in the United States. The addition of a second Bt protein provided increased control of bollworm (Stewart et al. 2001, Jackson et al. 2004) and Bollgard II cotton still remains as an effective management tool (Kerns et al. 2018). The addition of a second toxin (gene pyramiding) into Bt cotton was also intended to delay the development of resistance. The Bt proteins included in a pyramid ideally would not share the same site of action, interacting with different binding sites in the midgut, thus allowing for the killing of insects that have developed resistance to one of the proteins (Gould 1998). Binding assays with radiolabeled Cry1Ac and Cry2Ab revealed that these toxins have different high-affinity binding sites in *H. zea* brush border membrane vesicles (Hernandez-Rodriguez et al. 2008), thus suggesting that Cry1Ac and Cry2Ab could conjunctively delay the development of Bt resistance. However, the utility of these pyramided Bt traits is limited due to the inherent variability of susceptibility of *H. zea* populations to Cry1Ac (Luttrell et al. 1999) and the initial release of Bt cotton expressing only Cry1Ac.

Release of Cry1Ac in Bollgard seven years before the release of Bollgard II provided selection pressure for resistance to Cry1Ac before and while pyramided Bt traits were commercialized (Ali et al. 2006, Luttrell et al. 1999). Furthermore, Welch et al. (2015) found weak, but significant cross-resistance of Cry1Ac and Cry2Ab in assays with *H. zea*, suggesting possible shared low affinity binding sites or a possible resistance mechanism unrelated to binding. Examples of resistance mechanisms in lepidopteran species conferring cross-resistance to toxins with different binding sites include interference with proteolytic processing of protoxins (Oppert 1999), toxin

degradation (Shao et al. 1998), toxin sequestration (Gunning et al. 2005), and rapid recovery of the midgut epithelium (Forcada et al. 1999). However, these resistance mechanisms are less common and often result in lower levels of resistance compared to resistance mechanisms associated directly with reduced high affinity binding (Ferre and Van Rie 2002). Following the commercial release of Bollgard II, Bt cotton expressing Cry1F + Cry1Ac (WideStrike®, Dow AgroSciences) and Cry1Ab + Cry2Ae (TwinLink, Bayer CropScience) were also made commercially available.

The first Bt cotton expressing three Bt traits (WideStrike 3®, Dow AgroSciences) has been commercially available since 2014. In addition to expression of both Cry1F and Cry1Ac, this cotton also expresses Vip3Aa19. Vegetative insecticidal proteins (Vip) differ from Cry proteins in that they are expressed by *B. thuringiensis* prior to sporulation and secreted across the cell wall when the bacterium is in the vegetative stage of development (Estruch et al. 1996). Estruch et al. (1996) hypothesized that Vip3A proteins would have a novel mechanism of action compared with Cry proteins due to the lack of structural homology between the two different types of proteins (Chakroun et al. 2016). However, despite the lack of structural homology, Vip3A proteins are believed to exert toxicity through a sequence of events similar to the Cry proteins: proteolytic processing, passage across the peritrophic matrix, binding to receptors associated with the midgut epithelium, and the development of pores (Lee et al. 2003, Chakroun et al. 2016). Nevertheless, Vip3Aa19 was demonstrated to act independent of the Cry proteins, thus it was deemed an ideal candidate for pyramiding with the Cry proteins (Kurtz 2010, Levine et al. 2016).

Bt cotton expressing Cry1Ac + Cry2Ab + Vip3Aa19 (Bollgard III®, Monsanto Co.) and Cry1Ab + Cry2Ae + Vip3Aa19 (TwinLink Plus®, Bayer CropScience) were commercially released following the release of WideStrike 3. This third generation of Bt cotton technologies improved control of bollworm compared with the second generation of Bt technologies (Kerns et al. 2018). However, Yang et al. (2019) reported a *H. zea* population collected from Leptra® corn in Texas (Cry1Ab + Cry1F + Vip3A, Pioneer Hi-Bred) with reduced susceptibility to Vip3A. Both Cry1Ab and Cry1F have limited efficacy against *H. zea* (Buntin 2008), and field resistance to Cry1Ac and Cry1A.105 in corn has been reported (Reisig et al. 2018, Dively et al. 2016), leaving Vip3A as the only highly effective Bt toxin expressed by Leptra corn for *H. zea* management (Yang et al. 2019). Consequently, the utility of Bt pyramids containing Vip3A in cotton for delaying resistance development in *H. zea* field populations is controversial given the initial lack of efficacy of some Bt proteins, the prior development of resistance, and deployment of the same or similar toxins in Bt field corn.

High Dose/Refuge Strategy

A high dose/refuge strategy has long been suggested as a resistance management strategy for Bt corn and cotton (Gould et al. 1998, Ostlie et al. 1997, US EPA 2001). The US EPA Scientific Advisory Panel on Bt Plant-Pesticides defines a high dose as concentration of toxin that is at least 25 times higher than the LD_{99} of a susceptible strain (Gould 1994, US EPA-SAP 1998). Alternatively, it has been suggested that a high dose should be high enough to kill 95% of heterozygote resistant allele carriers (Andow and Hutchison 1998, US EPA 2001) or 50 times the concentration needed to kill 50% of susceptible larvae (Caprio et al. 2000). In essence,

the goal of a high dose is to eliminate the heterozygote carriers of rare resistance alleles, leaving only a small amount of homozygous resistant individuals. Additionally, incomplete resistance (Tabashnik and Carriere 2007) and fitness costs to resistance (Gassmann et al. 2009) have both been attributed to the success of Bt refuges by limiting the ecological advantages of resistant homozygous insects (Huang et al. 2011).

In supplement to a high Bt dose within transgenic plants, the use of non-Bt refuges is intended to serve as a resource of Bt-susceptible individuals that can mate with resistant individuals, thus diluting the frequency of resistance alleles. Due to the polyphagous nature of *H. zea*, wild host plants have also been proposed as potential Bt refuges (Jackson et al. 2008), and indeed, relying only on 'natural refuge' is allowed for pyramided Bt cotton technologies in the United States. Mandatory structured refuges, such as those required for corn in cotton growing areas of the U.S., can be implemented as either blocks or strips of Non-Bt crops (Ostlie et al. 1997, US EPA 2001). Alternatively, a refuge could consist of mixtures of non-Bt and Bt seed (i.e., refuge-in-the-bag, RIB, for corn) (Onstad et al. 2011). This method can potentially mitigate issues regarding grower refuge compliance, however, there are concerns that cross-pollination of non-Bt corn with Bt corn plants in a mixed refuge could result in increased selection for heterozygote resistance allele carriers via exposure to low doses of Bt proteins within the ear (Yang et al. 2014).

Unfortunately, *H. zea* challenges the criteria that ensure the success of the high dose/refuge management strategy. As previously discussed, *H. zea* has historically exhibited an inherit tolerance to some of the Cry proteins, and in at least parts of the U.S., it has been suggested to have developed field resistance to all the Cry proteins

(i.e., Cry1 and Cry2) expressed in Bt cotton or corn (Yang et al. 2017, Yang et al. 2018, Kerns et al. 2018, Kerns et al. 2019). Furthermore, the United States Environmental Protection Agency has indicated that corn producing Vip3A protein alone does not express a high-dose for management of *H. zea* (US EPA 2009). Additionally, the overwintering of bollworm in the southern United States and its movement from corn to cotton expressing similar Bt proteins over the course of the growing season allow for increased selection pressure that may also limit benefits provided by the high dose/refuge management strategy (US EPA 2001, Von Kanel et al. 2016).

The dose that a bollworm is exposed to in a Bt cotton field is dependent on environmental factors and larval behavior. Expression of Bt proteins in cotton has been found to vary spatially among different plant structures (Sivasupramaniam et al. 2014, Willrich Siebert et al. 2009) and temporally as the plant ages (Adamczyk et al. 2001, Kranthi et al. 2005). Abiotic stressors such as high temperature (Chen et al. 2005), nitrogen deficiency (Pettigrew and Adamczyk 2006, Coviella et al. 2002), high soil salinity, waterlogging (Luo et al. 2008), drought stress (Martins et al. 2008), and elevated CO_2 (Coviella et al. 2002) have all been suggested to negatively impact the concentration of Bt proteins in cotton tissues as well. Bollworm larvae have exhibited a preference for untreated meridic diet over diet that was treated with Cry1Ac (Gore et al. 2005), suggesting some degree of feeding avoidance. Likewise, Gore et al. (2002) suggested that bollworms placed on Cry1Ac-expressing cotton avoided areas with high concentrations of Cry1Ac and moved lower into the canopy than bollworms that were placed on non-Bt cotton. Conversely, this phenomenon was not observed when bollworms were placed on Bt cotton expressing Cry1Ac + Cry1F (Jackson et al. 2010).

An inconsistency of larval behavior for *H. zea* on different Bt technologies may complicate management decisions such as the need for supplemental insecticide applications. Larval Bt avoidance could also have implications for resistance management considering that larvae could select to feed on plant structures containing lower doses of Bt proteins, thus increasing opportunities for survival of individuals carrying resistance alleles.

Resistance Monitoring

Transgenic Bt corn and cotton have been widely adopted in the United States, with 80% of corn and 89% of cotton acres planted to Bt varieties (USDA-NASS 2020). The extensive adoption of Bt technologies as an insect management tool could place high selection pressure on target insect populations and result in the development of resistance. This highlights the importance of effective resistance management plans and the need to monitor for resistance to evaluate the success of resistance management plans that have been implemented. Various definitions of resistance are discussed in Huang et al. (2011), and much is still disputed regarding the definition of resistance. The Insecticide Resistance Action Committee states that an insect population is defined to have developed field resistance when the selection of a heritable characteristic has resulted in the “repeated failure of an insecticide product to provide the intended level of control when used as recommended” (IRAC 2010). Alternatively, Tabashnik et al. (2014) defines practical resistance as “field-evolved resistance that reduces pesticide efficacy and has practical consequences for pest control”.

Huang (2006) explains that a successful resistance monitoring program should be able to estimate the initial Bt resistance allele frequencies in field populations and detect early increases in Bt resistance allele frequencies long before field control failures occur due to the exponential development of resistance in field populations as resistance allele frequencies increase (Roush and Miller 1986). Bioassays for monitoring Bt resistance monitoring typically consist of exposing larvae to meridic diet treated with Bt proteins or plants containing Bt toxin to detect resistant individuals (Huang 2006). Different bioassay techniques that have been historically utilized include the exposure of larvae to diagnostic doses of toxin (i.e., LC_{50} or LC_{99}) through F0, F1, or F2 screening (Downes et al. 2016). F0 screens allow for the direct testing of field populations and require less labor and resources than other screens (Roush and Miller 1986, Downes et al. 2016). However, this screening method is unable to efficiently detect rare recessive alleles (Andow and Ives 2002). F1 screens involve crossing a field collected population with a homozygous resistant strain and screening the F1 progeny for resistance, with 50% of F1 progeny being homozygous carriers of the resistance allele if the field collected parent was a homozygous carrier (Gould et al. 1997). Unfortunately, this method requires that both parental strains have the same resistance alleles and that a resistant strain be established prior (Huang 2006). F2 screens are intended to generate isofemale lines that produce 1/16 of progeny that are homozygous carriers of a rare resistance allele (Andow and Alstad 1998). This screening method is effective for detecting rare resistance alleles (Huang 2006) and allows for the development of lab resistant strains that can be utilized in F1 screens (Downes et al. 2016).

The resistance monitoring bioassay surveys that have been traditionally utilized are both labor intensive and expensive (Huang 2006), creating logistical restrictions to long-term monitoring efforts. Reisig et al. (2018) provided evidence of practical resistance of *H. zea* to Cry1Ac in North Carolina by combining empirical data from an adaptation of F0 screening with observational data from cotton field surveys. The F0 screening consisted of dose-response assays that exposed field-collected populations to a series of doses to generate an LC_{50} that could be compared with the LC_{50} of a susceptible laboratory strain (i.e., resistance ratio) (Vennette et al. 2002). This method is inexpensive and less labor intensive (Huang 2006), therefore allowing for long-term monitoring of resistance with limited logistical restraints. Similar resistant monitoring bioassays conducted on *H. zea* populations collected from various locations in the midsouthern United States have suggested the development of substantial levels of resistance to Cry1Ac and Cry2Ab, and thus, concerns have arisen over increasing reliance and selection pressure for resistance on Vip3A (Kerns et al. 2019, Yang et al. 2019). However, these bioassays may not be indicative of how a population will perform in the field when exposed to a suite of toxins in pyramided Bt cotton or corn tissue. Anilkumar et al. (2008) demonstrated that an activated Cry1Ac-resistant *H. zea* strain exhibited only slight cross-resistance to a protoxin form of Cry1Ac, thus suggesting the form of a Bt protein used in an assay can have a substantial impact on the results of an assay. Additionally, the inherently wide range of Cry1Ac susceptibilities exhibited by *H. zea* field populations further complicate the development of field-relevant resistance monitoring methodologies. It has been demonstrated that the susceptibility of a laboratory susceptible strain that has been crossed with field collected populations can

vary substantially differ from the susceptibility of a laboratory susceptible strain that has not been out-crossed for an extended period of time (Anilkumar et al. 2008). Consequently, the proper methodologies required to confirm the field-evolved resistance of *H. zea* to the Bt proteins expressed in transgenic crops is controversial (Tabashnik et al. 2008, Moar et al. 2008). Bioassays using Bt cotton (Little et al. 2017) and corn leaf tissue (Kaur et al. 2019) have been suggested as a way to better expose larvae to field realistic forms of Bt proteins. However, secondary metabolites found in cotton have been found to increase (Anilkumar et al. 2009) or decrease (Olsen et al. 2000) the perceived toxicity of Cry1Ac in bioassays. Leaves are not preferred feeding sites of bollworms (Farrar and Bradley 1985), and nutrition, Bt proteins, and secondary metabolites are all believed to affect larval behavior (Orpet et al. 2015, Anilkumar et al. 2009, Gore et al. 2004, Reese et al. 1981). Therefore, alternative cotton plant structures may be more ideal for Bt resistance bioassays.

As previously discussed, the concentration gradient of Bt proteins expressed by transgenic Bt cotton has had varying effects on bollworm larval behavior dependent on the particular Bt technology that larvae are exposed to (Gore et al. 2002, Wilrich Siebert et al. 2009). These inconsistencies in larval behavior have led to deviations from standardized scouting methods by some pest advisors in favor of methods that they believe are more suitable for Bt cotton. These alternative methods may involve focusing on lower regions of the canopy, on small bolls, or on bloom tags (floral remnants) of bolls rather than the traditional, top-down scouting methodology. Consequently, the currently recommended treatment thresholds may not be suitable when making treatment decisions based on modified sampling procedures. Ideally, there would be a

standardized method of scouting and making insecticide treatment decisions that would be broadly suitable for Bt and non-Bt cotton varieties. Better understanding of how Bt technologies may affect the distribution of *H. zea* larvae and their damage is needed to advance the development of this standardized scouting methodology.

Nutrition, secondary metabolites, and Bt protein concentration can influence bollworm behavior and their survival on Bt cotton. This can have implications for how Bt cotton is scouted and how resistance is perceived in plant tissue bioassays. Further investigation of how these factors and their interactions affect *H. zea* larvae could lead to improved Bt cotton scouting methodologies and resistance monitoring bioassays.

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Chapter I. Impact of Various Bt Cotton Traits and the Application of an Insecticide on the Within Plant Distribution of *Helicoverpa zea* (Lepidoptera: Noctuidae) Larvae and Injured Fruiting Structures

Abstract

Previous studies have indicated that the expression of Bt insecticidal proteins in cotton can have a significant influence on the behavior of bollworm, *Helicoverpa zea* (Lepidoptera: Noctuidae). This suggests that the particular Bt trait that is associated with a cotton variety may need to be considered when determining the most ideal scouting methods to utilize for bollworm. Non-Bt, WideStrike, and Bollgard II cotton varieties were planted and either treated with an insecticide or left untreated. The presence of *H. zea* feeding injury and larvae were recorded according to location in the canopy and type of floral structure where found. Results indicated no significant differences in the distribution of larvae or damaged structures between the different cotton varieties, and insecticide treatment also had minimal impact. Larval sizes in different portions of the canopy suggested that larvae tended to move towards the middle of the canopy as they aged. Differences in larval behavior between Bt cotton technologies appear to have a more substantial effect on how quickly larvae move to preferred feeding sites rather than their preference for particular feeding sites. This study suggests that scouting methods could be standardized regardless of the presence or lack of a Bt cotton trait in a cotton variety, or whether a previous insecticide application was made or no insecticide was applied. Focusing scouting efforts on the middle portion of the canopy could increase the detection of small larvae and 'fresh' injury while being less influenced by previous insecticide applications.

Key Words: cotton, *Helicoverpa zea*, Bt cotton, larval distribution

Introduction

Helicoverpa zea (Lepidoptera: Noctuidae), commonly referred to as bollworm or corn earworm, has historically been considered a major pest of cotton in the United States (Luttrell 1994). Female moths have a preference for oviposition on host plants while they are flowering, thus *H. zea* is often a late season pest of cotton (Johnson et al. 1975). Typically, small larvae feed on small squares in the upper canopy before they increase in size and begin to feed lower in the canopy on larger fruiting structures such as bolls (Wilson et al. 1980, Reese et al. 1981, Farrar and Bradley 1985). Transgenic Bt cotton expresses one or more insecticidal proteins from the bacterium *Bacillus thuringiensis* to provide control of important lepidopteran larvae (Fleming et al. 2018, Kerns et al. 2018). The prominence of *H. zea* as a pest in cotton increased following the widespread adoption of Bt cotton as a standard insect management practice, in part because it is inherently less susceptible to the Bt proteins expressed in Bt cotton compared with the tobacco budworm, *Chloridea virescens* (Lepidoptera: Noctuidae) (Luttrell and Jackson 2012).

The application of a supplemental insecticide to Bt cotton is sometimes necessary to maintain adequate management of bollworm despite the substantial benefit the technology provides as a management tool (Reisig et al. 2019). Consequently, the scouting of Bt cotton for the presence of bollworm remains an important management tool. Previous studies suggest that the expression of Bt proteins in cotton plant tissues can significantly impact the behavior and plant canopy distribution of *H. zea* larvae (Gore et al. 2002, Gore et al. 2003, Jackson et al. 2010). Several factors have been identified as variables that can influence the behavior of *H. zea*

larvae in cotton. The concentration of Bt proteins in Bt cotton varies both spatially and temporally (Kranthi et al. 2005, Sivasupramaniam et al. 2014), and *H. zea* larvae have exhibited a preference for diets containing lower concentrations of Bt proteins (Gore et al. 2005). Bollworms have been observed to move lower into the canopy more rapidly in Bt cotton than in non-Bt cotton, possibly in response to the concentration gradient of Bt proteins throughout the plant (Gore et al. 2002). The window of time that an infestation occurs during the growing season has also been identified as a possible contributing factor to the varying behavior and canopy distribution of *H. zea* larvae. The vertical distribution of bollworm eggs was observed to favor the upper portion of the canopy later in the season but was more uniformly distributed earlier in the season, thus impacting the subsequent distribution of newly eclosed larvae (Braswell et al. 2019).

Since Bt cotton was first commercialized and as new Bt technologies have been introduced, some pest advisors have deviated from standardized scouting methods for bollworm in favor of methods they feel are more suitable for Bt cotton. This may involve focusing lower in the canopy, on small bolls, or the bloom tags (floral remnants) of bolls rather than more traditional systematic and top-down scouting. Consequently, currently recommended treatment thresholds may not be suitable when making treatment decisions based on modified sampling procedures. In addition, the various Bt technologies differ in their ability to control bollworm (Kerns et al. 2018), and thus, egg or larval thresholds should and often do differ among the technologies (e.g., Stewart and McClure 2020, Catchot 2020). These factors can create uncertainty and confusion when making insecticide treatment decisions for bollworm in systems where multiple Bt cotton technologies are deployed. This uncertainty is further compounded where *H. zea*

is developing resistance to some Bt toxins present in cotton (Reisig et al. 2018, Kerns et al. 2019, Yang et al. 2019).

Ideally, there would be a standardized method of scouting and making insecticide treatment decisions that would be suitable for Bt and non-Bt cotton varieties. An important step in the process of identifying the optimal scouting method is better understanding how Bt technologies affect the distribution of *H. zea* larvae and damage within the canopy of cotton which is the primary focus of this study.

Materials and Methods

Experimental Design

In 2018, eight row main plots of non-Bt Phytogen 425 RF (Corteva Agriscience, Indianapolis, IN), Phytogen 444 WRF (WideStrike, Cry1F + Cry1Ac, Corteva Agriscience, Indianapolis, IN), and Deltapine 1646 B2XF (Bollgard II, Cry1Ac + Cry2Ab, Bayer CropScience, St. Louis, MO) cotton varieties were planted within a randomized complete block design with four replications on 12 June in Jackson, TN. It was expected that these varieties would provide variable bollworm infestation levels owing to the presence (WideStrike, Bollgard II) or lack of Bt traits, and that these Bt traits may also affect the behavior of *H. zea* larvae. Row spacing was 97 cm, plots were 12 m long, and 13.3 seeds were planted per m row. Main plots were divided into four row sub-plots that were either treated or not treated with a foliar application of chlorantraniliprole (60 g ai/ha, Prevathon, FMC Corporation, Philadelphia, PA). This application was made on 21 August once *H. zea* larvae, consisting primarily of small larvae, were detected in the field at treatment levels.

The exact same experimental design, varieties, row spacing and planting rate was used in 2019, but the experiment was duplicated at multiple locations. Cotton was planted on 30 April, 16 May, and 4 June in College Station, TX, Tillar, AR, and Jackson, TN, respectively. Plot length varied from 12-14 m. In 2019, lambda-cyhalothrin (35.7 g ai/ha, Warrior II, Syngenta Corporation, Wilmington, DE) was used rather than chlorantraniliprole to allow for a greater post-treatment survival of bollworm. The insecticide application was made on 17 July in Texas, 24 July in Arkansas, and 15 August in Tennessee.

Sampling Procedures

In 2018, sampling was performed on 26 August. In 2019, samples were taken on 22 July, 30 July, and 20 August in Texas, Arkansas, and Tennessee, respectively. In both years, sampling for *H. zea* larvae and injury was done when cotton was near physiological cutout (i.e., 4-5 nodes above white flower [NAWF]), and thus plants had a near maximum number of total nodes and ample numbers of squares, blooms, and bolls of various sizes.

After a preliminary assessment, subplots treated with chlorantraniliprole in 2018 were not sampled because this application effectively reduced the number of *H. zea* larvae and injury levels to negligible levels. All subplots were sampled in 2019. The center two rows of subplots were sampled by selecting five consecutive plants from three randomly chosen spots. These plants were cut at the base of the plant and carried to the edge of the field. However, spindly or grossly atypical plants were avoided because they would make mapping the location of larvae and injury difficult.

Portable tables and tents were placed at the field edge, and the presence of *H. zea* larvae and injury for each of the 15 plants from a subplot were mapped immediately following removal from the field.

Mapping consisted of recording the node where a larva or injured floral structure was found. Larvae were categorized as either small (1st and 2nd instar), medium (3rd and 4th instar), or large (5th instar or larger). A floral structure was considered injured if the square or boll 'wall' had been penetrated. Injury to flowers also included obvious feeding signs on the petals. We categorized whether the larva or injury was found on a square, candle square, white flower, pink flower, bloom tag boll, small boll, or boll. A candle square is the last stage of development of a square before it opens as a flower, thus all squares in the candle stage were categorized as "candle squares" and all other squares in prior stages of development were categorized together as "squares". A cotton flower only persists for one day as a "white flower", after which the white petals turn pink and begin to wither. "Pink flowers" were those that retained some moisture and pink coloration, typically for 2-4 days after flowering. After pink flower, the dried bloom remnants either fall off the boll or remain stuck to the tip of the boll (i.e., bloom tag). Bolls that retained a bloom tag were categorized as "bloom tag bolls" and bolls that were similar in size but had no bloom tag were categorized as small bolls. Any larger bolls were categorized as "bolls".

Analyses

The cumulative number of larvae (by larval size) and the total amount of injury (by floral structure) were calculated for each subplot. For analyses, larval location and

injury were categorized by canopy level (top, middle, bottom). The top five nodes of plants were designated as the top canopy, nodes six through nine were designated as the middle canopy, and nodes below the ninth node were considered the bottom canopy. After preliminary analyses, it was decided to more coarsely categorize larval location and injury for floral structures as square (square and candle square), flower (white flower and pink flower), or boll (bloom tag boll, small boll, boll), rather than by the finer categorizations chosen when the data were collected. This was done because some of the sample sizes for the finer categorizations were too small to make any meaningful comparisons between. Similarly, low numbers of larvae and injury were observed in Bollgard II cotton plots, and initial analyses indicated no significant differences in the distribution of larvae or injury between Bollgard II and WideStrike cotton. Thus, Bollgard II and WideStrike cotton plots were labeled as a single, indistinguishable “Bt” treatment for all analyses to increase statistical power.

To normalize the data, log transformations were done before analyzing with GLIMMIX procedures (α=0.05, SAS ver. 9.4, SAS Institute, Cary, NC). Fixed effects included in the statistical models included Bt trait (non-Bt and Bt), insecticide treatment (treated or not), canopy level (top, middle, bottom), floral structure (square, flower, boll), larva size (small, medium, large) and all their interactions. Depending on the comparisons being made, models did not include all fixed effects, and variations of these fixed effects are specified in Table 1. Random effects in the models included location, appropriate interactions between locations and other effects, and replication as a nested effect within other model effects (Table 1).

For data collected in 2019, the distribution of injured structures throughout the canopy or between different floral structure types was analyzed using two separate models (Table 1; Models 1 and 2). The distribution of larvae based on canopy level and floral structure type in 2019 was also analyzed as two separate models (Table 1; Models 3 and 4). Fixed effects were the same as the first two models that were previously discussed, however, no three-way interaction was included in model 4 due to failure of the model to converge.

Data collected in 2018 and 2019 were analyzed together to evaluate the distribution of injured structures within the canopy and between types of floral structures (Table 1; Models 5 and 6). Insecticide treatment was excluded as a main effect from all models that analyzed data from 2018 because no data on insecticide effects was collected that year due to low survival of larvae in treated plots. A model to analyze the number of observed larvae distributed between different canopy levels was also constructed from compiled 2018 and 2019 data (Table 1; Model 7). Another model was constructed to analyze the distribution of larvae between different floral structures, however, only trait and floral structure type were included in the model as main effects so that the model would converge (Table 1; Model 8). The data from 2018 and 2019 was partitioned by canopy level (top, middle, bottom) and included in three separate models to evaluate larva size distribution in each portion of the canopy (Table 1; Models 9, 10, and 11).

Results

In 2018, no *H. zea* larvae and very little injury was found in preliminary samples of non-Bt cotton that were treated with chlorantraniliprole. Therefore, subplots treated

with this insecticide were not sampled. Consequently, data was unbalanced across years, and the results are presented either across years or for 2019 alone as appropriate for the statistical comparisons of interest. The trial conducted in 2018 was the most heavily infested test despite being the only location tested that year. Overall bollworm infestation levels would be considered moderate and somewhat lower than might normally be observed. The average total number of injured floral structures observed on 15 plants in non-Bt plots that were not treated with an insecticide was 38.25 ± 12.44, 14.0 ± 3.72, 14.0 ± 2.68, and 24.25 ± 1.49 for Tennessee (2018), Tennessee (2019), Arkansas, and Texas respectively.

Vertical Distribution of Injury and Larvae in the Canopy

As expected, the non-Bt cotton variety had considerably more injured fruiting structures than the Bt varieties, regardless of whether data were combined across years or not (Table 2). The application of a pyrethroid insecticide in 2019 did not significantly reduce the total amount of injury caused by *H. zea* larvae (Table 2). Less injury was observed in the bottom portion of the canopy compared with the middle and upper portions, and again, this pattern was similar when data were combined across years or not (Table 2). The effects of canopy level and insecticide were found to have a significant interaction (Table 2). Injury in the upper canopy was significantly reduced by approximately 54% in plots that received a pyrethroid treatment (Figure 1). In contrast, there was a slight, but not statistically significant, increase in the mean number of injured structures in the bottom portion of the canopy when a pyrethroid insecticide was applied. No other two-way or three-way interactions were observed.

Larval numbers were low compared with the numbers of injured floral structures, but similar to injury, the vast majority of larvae were found in the non-Bt cotton, and like injury to fruiting structures, this was true regardless of whether the data were analyzed across years or not. (Table 3). There was no significant difference between the number of larvae observed in cotton treated with a pyrethroid and cotton that was not treated (Table 3). Canopy level had a significant effect on the amount of observed larvae (Table 3). Most larvae were found in the top and, in particular, the middle portion of the canopy. Interactions were not observed (Table 3).

Trait did not have a significant influence on the number of larvae observed in each individual portion of the canopy (Table 4). However, the trend in each part of the canopy matched the overall observation (Table 3) of fewer larvae in Bt cotton than in non-Bt cotton. Mostly small and medium sized larvae were found, regardless of canopy level, with more medium sized larvae observed in the middle canopy than small and large larvae (Table 4). No interaction between trait and larval size was observed in any portion of the canopy (Table 4).

Distribution of Injury and Larvae Among Floral Structures

As seen with the previous analyses, more injured fruiting structures were observed in non-Bt cotton compared to cotton with Bt traits, and no significant difference in the total number of injured structures was observed between plots that were treated with a pyrethroid insecticide and plots that were not (Table 5). Squares and bolls were the most commonly observed injured structures (Table 5). No significant interactions among the main effects were found (Table 5).

Also as seen with the previous analyses, larvae were more common in the non-Bt cotton compared with cotton having Bt traits, and there was no significant difference in the number of larvae found in plots that were treated with a pyrethroid insecticide compared with those not treated (Table 6). Significantly more larvae were found on bolls than squares or flowers when analyses were conducted across both years or for 2019 alone (Table 6). The mean number of larvae found on squares did not statistically separate from the mean number of larvae found on flowers. Two-way and three-way interactions of main effects on larval numbers were not significant (Table 6).

Discussion

Non-Bt cotton consistently had more damaged structures and larvae in all statistical comparisons. Thus, the Bt technologies provided some plant protection despite reports of resistance to multiple Bt proteins in the area where these studies were performed (Kerns et al. 2019). Chlorantraniliprole provided excellent control of *H. zea* during 2018, and thus, data were not collected in plots treated with chlorantraniliprole because no larvae were present. Insecticide products containing chlorantraniliprole are now widely used to control *H. zea* in cotton because they provide effective and lasting control (e.g., Steckel and Stewart 2012). Moreover, Adams et al. (2016) did not detect meaningful levels of *H. zea* resistance to chlorantraniliprole in the midsouthern United States. In contrast, increasing *H. zea* resistance to pyrethroid insecticides has been well documented in the last decade (Musser et al. 2017, Reisig et al. 2019). Treatment with a pyrethroid did not significantly reduce the overall number of injured floral structures or larvae observed in our trials, on any of the Bt or non-Bt varieties that were tested. However, the amount of damaged structures in the upper

portion of the canopy was reduced after treatment with a pyrethroid. The pyrethroid did not significantly affect the number of larvae in the upper canopy, although there was a slight trend indicating a marginal reduction. Presumably, there is better insecticide coverage in the upper canopy, resulting in better larval mortality and a reduction of floral injury. However, it is also possible that the larvae were sub-lethally sickened or had aversion to the pyrethroid insecticide, and thus, the reduction of injury observed in the upper canopy was an anti-feeding response (Hannig et al. 2009). The test in 2018 experienced a rapid onset of bollworm, whereas the tests in 2019 had a lower and more gradual onset of pest pressure. This trickling of bollworms in 2019 made it difficult to time a single pyrethroid application, and likely contributed to the poor control observed with the pyrethroid insecticide.

The higher proportion of small larvae found in the top portion of the canopy indicates that moths were more likely to oviposit in this portion of the canopy. This finding is not unlike other findings from previous studies (Farrar and Bradley 1985, Gore et al. 2002, Torres and Ruberson 2006). Because samples were collected near physiological cutout, flowers were present in the top portion of the canopy (Bourland et al. 2001). Bollworm moths are drawn to flowers as a source of nectar (Fitt 1989), and thus they may be more likely to oviposit in areas of the canopy where flowers are present (Braswell et al. 2019). Furthermore, the presence of small larvae on small bolls, and especially small bolls with a bloom tag, could be an indicator of oviposition on flowers. In plots that were not treated with an insecticide, across 2018 and 2019, 60.7% of small larvae were found on bolls, and 55.9% of those larvae were found to be on bolls with a bloom tag or small bolls that would have recently shed a bloom. This is

substantial given that bolls classified as small bolls or bolls with a bloom tag comprised 30.9% of injured bolls.

The middle portion of the canopy contained a high proportion of medium larvae, which would support previous observations of downward larval movement on cotton plants (Farrar and Bradley 1985, Gore et al. 2002, Braswell et al. 2019). Larvae in early instars feed on squares and begin to feed on bolls after increasing in size (Farrar and Bradley 1985). Bolls in the middle portion of the canopy constitute a sizeable portion of the overall lint yield (Ritchie et al. 2007), thus downward larval movement may have been influenced by preference for or sheer numbers of susceptible floral structures. Floral structures in the upper portion of the canopy start decreasing in quantity as the plants mature and larvae feed, thus larvae would be required to move downward to reach more food sources (Braswell et al. 2019). Fewer larvae and damaged fruiting structures were observed in the bottom portion of the canopy. This was likely partly due to the ovipositional preferences of moths that were previously discussed. At the time of sampling, the bolls in the bottom portion of the canopy would have matured enough to make it difficult for small larvae to successfully establish due to the inability to penetrate the boll wall (Benedict et al. 1997).

The distribution of larvae and injury did not significantly differ between different cotton varieties, regardless of the presence of a Bt trait or not. Thus, our results suggest that it would be appropriate to use standardized scouting methods in Bt and non-Bt cotton varieties. Results from Gore et al. (2002) showed that larval behavior in Bt cotton may be altered due to the avoidance of high concentrations of Bt proteins. Small but statistically insignificant trends observed in this study suggest the same phenomenon,

with a higher proportion of larvae and injury occurring lower in the canopy of Bt cotton compared with non-Bt cotton. Had we had higher bollworm pressure, this effect may have been more pronounced. Similarly, this study did not see major effects of pyrethroid treatment on the distribution of larvae or injury to floral structures. Differences may have been more pronounced had a more effective insecticide been used, but pragmatically, this data indicates that changes in the distribution of larvae or injury are not substantial enough to justify different scouting procedures on non-Bt and different Bt cotton varieties or on insecticide treated or non-treated fields. These data would support that scouting efforts could be focused on the middle part of the canopy when cotton is flowering. This study found as much or more small larvae and injury in the middle part of the canopy. Based on our results and other research, focusing scouting efforts on the middle portion of the canopy should increase the detection of small larvae and 'fresh' injury and be less influenced by previous insecticide applications.

Not surprisingly, finding injury to floral structures was more common than finding larvae because one larva often feeds on multiple structures (Wilson and Gutierrez 1980). As is in practice today (e.g., Stewart and McClure 2020, Catchot 2020, Ring 2019), treatment thresholds in both non-Bt cotton and Bt cotton are based on larva counts and/or percent injury to fruiting structures. Given the discussion above, our data suggests sampling of pink flowers and small bolls (including bolls with bloom tags) would be an appropriate scouting method to detect bollworm infestations and make insecticide treatment decisions, at least when bollworm infestations are most likely to occur (at peak flowering and beyond). A recent study on non-Bt and multiple Bt cotton technologies indicated that insecticide management decisions based on injury to

squares or small bolls provided economic returns as high or higher than a more proactive and aggressive insecticide approach (Kerns et al. 2017). Insecticide recommendations based on the presence of bollworm eggs does not seem like a sustainable approach where multiple Bt cotton technologies are grown (or non-Bt cotton) because it would require different thresholds based on the efficacy of the technology, which would also be influenced by evolving levels of resistance to Bt toxins (e.g., Tabashnik and Carrière 2017) or difference in expression profiles among plant parts, varieties, or at different times of the season (Sivasupramaniam et al. 2014, Kranthi et al. 2005, Adamczyk et al. 2001, Carrière et al. 2018). Further research is justified, particularly under conditions of very high or early onset of bollworm infestation, however, standardizing insecticide application recommendations for bollworm in non-Bt and Bt cotton varieties appears to be a simple and appropriate approach.

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Appendix

Table 1. List of all main effects, interactions between main effects, and random effects that were included in each statistical model that was part of the analysis for this study.

Fixed and Random Effects for All Models			
Models	**Main Effects**	**Main Effect Interactions**	**Random Effects**
1	Trait Insecticide Canopy	All 2- and 3-way	Location, Location*Trait, Location*Insecticide, Location*Trait*Insecticide, Location*Trait*Insecticide*Canopy, Rep(Location*Trait*Insecticide)
2	Trait Insecticide Structure	All 2- and 3-way	Location, Location*Trait, Location*Insecticide, Location*Trait*Insecticide, Location*Trait*Insecticide*Structure, Rep(Location*Trait*Insecticide)
3	Trait Insecticide Canopy	All 2-and 3-way	Location, Location*Trait, Location*Insecticide, Location*Trait*Insecticide, Location*Trait*Insecticide*Canopy, Rep(Location*Trait*Insecticide)
4	Trait Insecticide Structure	All 2-way	Location, Location*Trait, Location*Insecticide, Location*Trait*Insecticide, Location*Trait*Insecticide*Structure, Rep(Location*Trait*Insecticide)
5	Trait Canopy	Trait*Canopy	Location, Location*Trait, Location*Trait*Canopy, Rep(Location*Trait)
6	Trait Structure	Trait*Structure	Location, Location*Trait, Location*Trait*Canopy, Rep(Location*Trait)
7	Trait Canopy	Trait*Canopy	Location, Location*Trait, Location*Trait*Canopy, Rep(Location*Trait)
8	Trait Structure	Trait*Structure	Location, Location*Trait, Rep(Location*Trait)
9, 10, 11	Trait Larval Size	Trait*Size	Location, Location*Trait, Rep(Location*Trait)

Table 2. Effect of Bt trait, foliar insecticide treatment, or canopy level on the mean observed damaged foliar structures in either 2018 and 2019 or 2019 alone.

Mean Damaged Foliar Structures				
Main Effect	**Treatments**	**2018+2019**	**2019**	
Trait	Non-Bt	17.73a	13.35a	
	Bt	4.14b	2.7b	
Insecticide	Treated	---	5.76a	
	Untreated	---	6.24a	
Canopy	Top	3.62a	2.32a	
	Middle	3.64a	2.62a	
	Bottom	1.77b	1.3b	
Type III Tests of Fixed Effects				
Year	**Main Effect**	**F-Value**	**df**	**P-Value**
2018+2019[a]	Trait	17.18	1, 3	0.0255
	Canopy	5.96	2, 12	0.0159
	Trait*Canopy	0.46	2, 12	0.6435
2019[b]	Trait	26.39	1, 2	0.0359
	Insecticide	0.24	1, 2	0.6744
	Canopy	7.01	2, 16	0.0065
	Trait*Insecticide	0.27	1, 2	0.6523
	Trait*Canopy	0.29	2, 16	0.7531
	Insecticide*Canopy	4.04	2, 16	0.0380
	Trait*Insecticide*Canopy	1.26	2, 16	0.3099

[a,b] Statistical Models 5 and 1 respectively (Table 1).

Table 3. Effect of Bt trait, foliar insecticide treatment, or canopy level on the mean observed number of *Helicoverpa zea* larvae in either 2018 + 2019 or 2019.

Mean Observed Larvae				
Main Effect	**Treatments**	**2018+2019**	**2019**	
Trait	Non-Bt	3.48a	2.31a	
	Bt	0.93b	0.36b	
Insecticide	Treated	---	0.84a	
	Untreated	---	0.99a	
Canopy	Top	0.56ab	0.29ab	
	Middle	0.99a	0.54a	
	Bottom	0.38b	0.18b	
Type III Tests of Fixed Effects				
Year	**Main Effect**	**F-Value**	**df**	**P-Value**
2018+2019[a]	Trait	19.51	1, 3	0.0215
	Canopy	5.28	2, 12	0.0227
	Trait*Canopy	0.96	2, 12	0.4088
2019[b]	Trait	25.77	1, 2	0.0367
	Insecticide	0.24	1, 2	0.6734
	Canopy	5.93	2, 16	0.0119
	Trait*Insecticide	0.45	1, 2	0.5723
	Trait*Canopy	1.86	2, 16	0.1880
	Insecticide*Canopy	0.77	2, 16	0.4773
	Trait*Insecticide*Canopy	0.17	2, 16	0.8417

[a,b] Statistical models 7 and 3 respectively (Table 1).

Table 4. Effect of Bt trait and larva size on the mean number of *Helicoverpa zea* larvae found in the top, middle, or bottom portions of the canopy in both 2018 and 2019 combined.

Mean Observed Larvae				
Main Effect	**Treatments**	**Top**	**Middle**	**Bottom**
Trait	Non-Bt	1.05a	1.59a	0.54a
	Bt	0.15a	0.63a	0.15a
Size	Small	0.33a	0.33b	0.11ab
	Medium	0.16ab	0.59a	0.17a
	Large	0.05b	0.18b	0.04b
Type III Tests of Fixed Effects				
Canopy	**Main Effect**	**F-Value**	**df**	**P-Value**
Top[a]	Trait	9.25	1, 3	0.0558
	Size	5.97	2, 108	0.0035
	Trait*Size	0.88	2, 108	0.4164
Middle[b]	Trait	9.66	1, 3	0.0530
	Size	6.35	2, 108	0.0025
	Trait*Size	0.95	2, 108	0.3897
Bottom[c]	Trait	1.91	1, 3	0.1387
	Size	2.24	2, 108	0.1103
	Trait*Size	0.82	2, 108	0.4432

[a,b,c] Statistical models 9, 10, and 11 respectively (Table 1).

Table 5. Effect of Bt trait, foliar insecticide treatment, or floral structure type on the mean observed damaged fruiting structures in either 2018 + 2019 or 2019.

Mean Damaged Floral Structures				
Main Effect	**Treatments**	**2018+2019**	**2019**	
Trait	Non-Bt	14.04a	11.7a	
	Bt	2.91b	2.49b	
Insecticide	Treated	---	5.01a	
	Untreated	---	5.85a	
Structure	Squares	4.92a	3.83a	
	Bolls	4.21a	2.74a	
	Flowers	0.47b	0.55b	
Type III Tests of Fixed Effects				
Year	**Main Effect**	**F-Value**	**df**	**P-Value**
2018+2019[a]	Trait	30.80	1, 3	0.0115
	Structure	14.30	2, 6	0.0052
	Trait*Structure	1.57	2, 6	0.2830
2019[b]	Trait	31.93	1, 2	0.0299
	Insecticide	0.69	1, 2	0.4930
	Structure	34.39	2, 16	<.0001
	Trait*Insecticide	0.12	1, 2	0.7612
	Trait*Structure	0.29	2, 16	0.7490
	Insecticide*Structure	0.40	2, 16	0.6770
	Trait*Insecticide*Structure	0.08	2, 16	0.9203

[a,b] Statistical models 6 and 2 respectively (Table 1).

Table 6. Effect of Bt trait, foliar treatment (lambda-cyhalothrin), or fruiting structure type on the mean number of observed *Helicoverpa zea* larvae in either 2018 + 2019 or 2019.

Mean Observed Larvae				
Main Effect	**Treatments**	**2018+2019**	**2019**	
Trait	Non-Bt	3.51a	2.43a	
	Bt	0.66b	0.36b	
Insecticide	Treated	---	0.78a	
	Untreated	---	1.08a	
Structure	Squares	0.46b	0.18b	
	Bolls	1.23a	0.65a	
	Flowers	0.23b	0.25b	
Type III Tests of Fixed Effects				
Year	**Main Effect**	**F-Value**	**df**	**P-Value**
2018+2019[a]	Trait	15.21	1, 3	0.0299
	Structure	6.39	2, 12	0.0129
	Trait*Structure	1.17	2, 12	0.3436
2019[b]	Trait	28.10	1, 2	0.0338
	Insecticide	0.93	1, 2	0.4361
	Structure	7.36	2, 18	0.0046
	Trait*Insecticide	0.02	1, 2	0.8894
	Trait*Structure	2.54	2, 18	0.1065
	Insecticide*Structure	0.29	2, 18	0.7506

[a,b] Statistical models 8 and 4 respectively (Table 1).

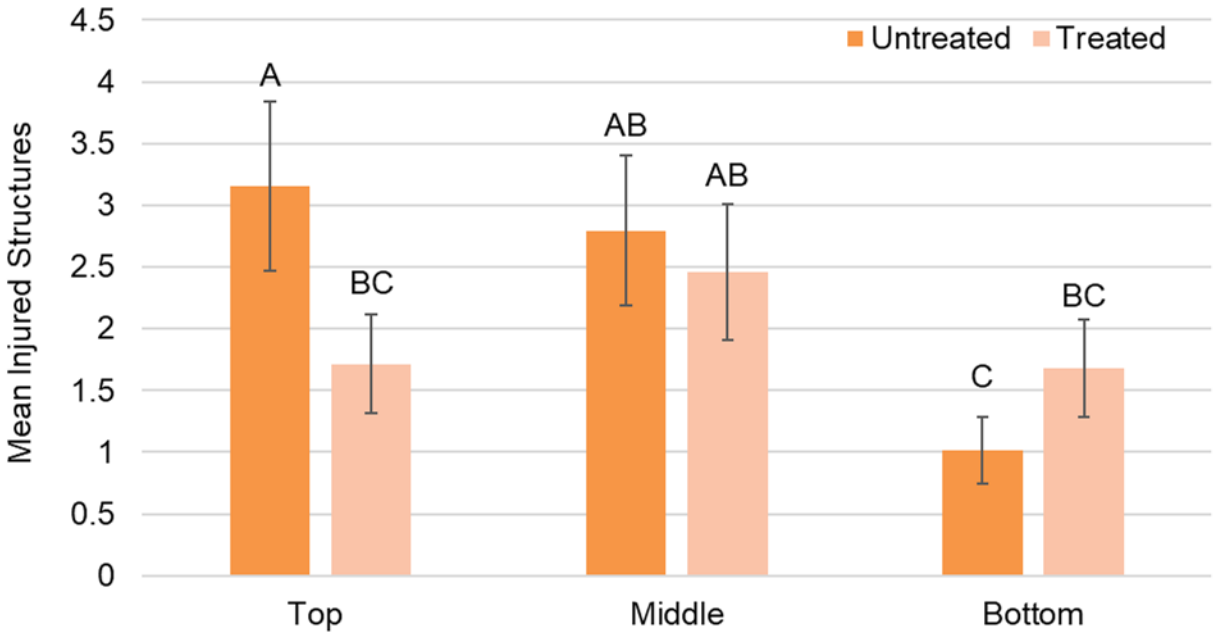

Figure 1. Effect of foliar application of lambda-cyhalothrin on the mean number of damaged floral structures observed in each portion of the canopy across all three cotton traits at all three locations in 2019 (f=3.63; df=2,8; p=0.0380).

Chapter II. Evaluation of Bt Resistance in *Helicoverpa zea* (Lepidoptera: Noctuidae) Strains Using Various Bt Cotton Plant Tissues

Abstract

Diet-overlay bioassays have suggested that *Helicoverpa zea* (Lepidoptera: Noctuidae) field populations may have developed resistance to some of the Bt insecticidal proteins that are constituents of the pyramids expressed in the second and third generation Bt cotton technologies. Unfortunately, these bioassays are not always a reliable indicator for how a seemingly resistant population will perform in an actual cotton field, thus leaf tissue bioassays have been suggested as a method to better assess field performance. However, bollworm larvae typically prefer to feed on floral tissue rather than leaf tissue, and an alternative cotton structure type may be more ideal for use in plant tissue-based bioassays. A series of Bt protein and Bt cotton plant tissue diet-overlay bioassays were conducted with laboratory susceptible (Benzon) and resistant (G13-RR, resistant to Cry1Ac, Cry2Ab, Cry1F) *H. zea* strains to determine if plant tissue overlays could detect resistance and which cotton plant structure type would be most ideal for use in bioassays. Leaves, squares, bolls, white flowers, and pink flowers were collected from non-Bt, Bollgard II, and WideStrike 3 cotton varieties during peak flowering, lyophilized, and ground into a fine powder for use in bioassays. Results suggested that lyophilized plant tissue-overlays were able to detect resistance and that white flowers were the most ideal structure type for use in bioassays. Non-Bt bolls and leaves substantially affected larval health and behavior, thus these tissues would confound results. White flower tissue overlays could potentially be used to supplement Bt protein overlays and provide an improved assessment of larval performance on Bt cotton technologies.

Key Words: Bt, bioassay, cotton, *Helicoverpa zea*

Introduction

Transgenic Bt cotton has been adopted as a major management tool for lepidopteran pests in the United States. The bollworm, *Helicoverpa zea* (Boddie) (Noctuidae), became a more predominant pest after the release of the first Bt cotton (i.e., Bollgard, expressing Cry1Ac) due to its higher tolerance to Cry1Ac compared with the tobacco budworm, *Chloridea virescens* (F.) (Noctuidae) (Luttrell and Jackson 2012). Since the first introduction of Bt cotton, traits for additional Bt toxins have been pyramided with Cry1Ac in various combinations including Cry1F, Cry2Ab or Cry2Ae, and more recently Vip3Aa19. Field-evolved resistance to Cry1Ac and Cry2Ab has been documented in *H. zea* populations, thus raising the concern for the development of widespread resistance (Tabashnik and Carrière 2017). More recent bioassays on bollworm populations from the mid-southern U.S. indicated substantial levels of resistance to the Cry proteins (i.e., Cry1A and Cry2A) but confirmed susceptibility to the Vip3A protein that is expressed by the most, recent-commercially available Bt cotton varieties (Yang et al. 2017, Yang et al. 2018, Kerns et al. 2019).

Resistance monitoring assays, however, may not be indicative of how a population will perform when exposed to Bt toxins in cotton or other crops, particularly when the Bt technologies may express multiple toxins. For example, Gould et al. (1995) established the YHD2 strain of *C. virescens* which exhibited levels of resistance approximately equal to 10,000-fold when reared on meridic diet incorporated with Cry1Ac. However, this strain was unable to survive when reared on Bt cotton plants (Tabashnik et al. 2003). It is possible that this laboratory resistant strain developed a form of resistance that would not be viable in the field or that the comparison of this

strain with highly susceptible laboratory strains yielded a resistance ratio that is not biologically relevant to the levels of Bt proteins expressed in transgenic crops. Thus, cotton leaf tissue assays have been proposed as a method to assess *H. zea* populations under conditions that are more ecologically significant than diet-based assays (Little et al. 2017).

Diet-based assays are typically limited to assessing only single Bt proteins. In contrast, the transgenic Bt cotton varieties available today express a suite of proteins, and larvae are simultaneously exposed to multiple Bt proteins in the field. The leaf tissue assays described by Little et al. (2017) allow for the collective assessment of the suites of Bt proteins that are available in Bt cotton rather than individual assessments of each Bt protein. Additionally, the form of the Bt protein that is used in an assay can impact how resistance is perceived in a population. A resistant *H. zea* strain established via selection with activated Cry1Ac, that is more similar to the truncated forms of Bt proteins in Bt cotton tissue, was only slightly cross-resistant to the protoxin form of Cry1Ac; thus suggesting that each form of the toxin would provide different results in resistance assays (Anilkumar et al. 2008). Therefore, assays that utilize plant tissue may provide Bt proteins in a form that is more biologically relevant than assays that utilize other forms of Bt proteins.

The concentration of Bt proteins can vary spatially among different plant structures of the cotton plant (Kranthi et al. 2005, Sivasupramaniam et al. 2014) and temporally throughout the growing season (Adamczyk Jr et al. 2001, Kranthi et al. 2005). Thus, the type of plant structure used in an assay and the physiological state of the plant when the structure was collected can influence the dose that larvae receive in

a plant-based assay (Carrière et al. 2018). Likewise, the concentrations of secondary metabolites in cotton plants can also vary both spatially and temporally (Zummo et al. 1984, Lege et al. 1992). Assays with bollworm larvae suggest that gossypol can interact synergistically with Cry1Ac, thus increasing the perceived toxicity of Cry1Ac in plant tissues (Anilkumar et al. 2009). In contrast, tannins have been found to reduce the toxicity of Cry1Ac to *Helicoverpa armigera* (Hubner) (Olsen and Daly 2000). Nutrition has also been identified as a variable that can influence the results of Bt resistance assays (Deans et al. 2016). For instance, Cry1Ac was more toxic to bollworm larvae that were fed a diet with a lower protein to carbohydrate ratio (Orpet et al. 2015b). Additionally, bollworm larvae are selective in their diet, not feeding indiscriminately regardless of the food source (Deans et al. 2015). Diet selectivity is not exclusive to nutritional attributes, however, and Bt proteins, secondary metabolites, and resistance associated with a particular insect population are all likely to influence larval feeding behavior (Gore et al. 2002, Gore et al. 2005, Anilkumar et al. 2009, Orpet et al. 2015a, Orpet et al. 2015b). Bt resistance assays can be influenced by larval feeding behavior given that Bt proteins must be ingested before they can have any physiological impact on the insect (Jurat-Fuentes and Crickmore 2017).

Using tissue-based assays could potentially assess the net resistance to an entire suite of toxins expressed in various Bt cotton technologies and better estimate larval performance when exposed to Bt proteins under field conditions. Further, plant tissues can generally be collected in large amounts and may preclude the need for producing purified Bt proteins to assess resistance. However, considering the points above, it is apparent that the kind of plant tissue used in resistance assays could

substantially influence the results. Terminal leaves contain a high concentration of Cry1Ac relative to other cotton plant structures (Greenplate 1999, Kranthi et al. 2005, Willrich Siebert et al. 2009, Sivasupramaniam et al. 2014), and they seem to be a logical and easily accessible tissue to utilize in assays (Little et. al 2017). Although bollworm larvae are known to feed on leaf tissue (Schmidt et al. 1988), they have a preference for floral structures (i.e., squares, flowers, bolls) (Farrar and Bradley 1985). Thus, other plant tissues should be evaluated for use in tissue-based assays for Bt resistance as they may have less negative influence on larval feeding behavior, better reflect the preferred larval diet, and be more sensitive in detecting actual levels of resistance. The primary objective of this research was to evaluate the utility of various Bt plant tissues in assessing levels of Bt resistance, in this case, with *H. zea* and cotton.

Materials and Methods

Purified Proteins and Plant Material

Purified Bt proteins were provided by Dr. Juan Luis Jurat-Fuentes (Department of Entomology and Plant Pathology, The University of Tennessee). These proteins were grown from recombinant strains of *Bacillus thuringiensis* (Cry1Ac and Cry2Aa) or *Escherichia coli* (Vip3Aa39) similar to the procedures described by Luo et al. (1999) and Chakroun et al. (2012). Cry1Ac and Cry2Aa proteins were trypsin activated, whereas Vip3Aa39 proteins were not trypsin activated, and thus these proteins were protoxins. All proteins were FPLC purified via ion exchange column, and stored at -80°C until needed. Cry2Aa proteins were used rather than Cry2Ab proteins due to difficulties in obtaining purified Cry2Ab proteins. These proteins have been reported to have a gene sequence homology of 89% (Dankocsik et al. 1990) and a shared binding site

(Hernandez-Rodriguez et al. 2008), thus it is likely that Cry2Aa proteins could be used to adequately detect Cry2Ab resistance. Likewise, Vip3Aa39 proteins have approximately 95% homology compared with Vip3Aa19 and Vip3Aa20, transgenic traits expressed in Bt cotton and corn, respectively.

Non-Bt Deltapine 1822 XF (Bayer CropScience, St. Louis, MO), Deltapine 1518 B2XF (Bollgard II, Cry1Ac + Cry2Ab, Bayer CropScience, St. Louis, MO), and Phytogen 330 W3FE (WideStrike 3, Cry1F + Cry1Ac + Vip3Aa19, Corteva Agriscience, Indianapolis, IN) were planted in the field on 4 June, 2019 in Jackson, TN. Plant structures including leaves, squares, white flowers, pink flowers, and bolls were collected from each variety on 20 August when cotton was near physiological cutout (i.e., 5 nodes above white flower [NAWF]). Ample numbers of bolls, squares, and blooms were present on the cotton at this stage of development. Leaves were collected from the third node from the top of the plant. Squares that were collected were between the match-head and midpoint stages of development (i.e., Ritchie et al. 2007). White flowers and pink flowers were also collected. A white flower persists for one day before it begins to whither and turn pink. Flowers that had a pink coloration and still retained moisture (i.e., 2-4 days after flowering) were considered pink flowers. The bolls collected were medium sized (i.e., 2-3 cm in diameter) and approximately 7-12 days old.

These plant structures were stored at -80°C until they were lyophilized and ground into a homogenous, fine powder using a coffee grinder (Fast Touch Electric Coffee Grinder; Solengen, Germany). 99% and 90% of the cotton plant powders could pass through 40 and 80 mesh sieves, respectively. For efficient processing of tissues, the inner fiber and seeds of bolls were discarded before being lyophilized; bracts were

removed from squares; stems were removed from leaves; and only the petals and external reproductive structures (i.e., style, stigma, filaments, anthers) of white and pink flowers were lyophilized. These plant tissues were stored at -80°C until used in bioassays.

Bioassays Using Purified Bt Proteins

Bioassays were performed to assess the susceptibility of *H. zea* larvae to Cry1Ac, Cry2Aa, and Vip3Aa39 Bt proteins. A *H. zea* strain, G13-RR, with known resistance to Cry1Ac and Cry2Ab (Fei Yang, per. comm.) was provided by Texas A&M University and utilized in these assays for evaluations. This strain was collected in 2018 from Bt corn (Cry1A.105 and Cry2Ab2) in Snook (TX) and established using an F2 screening method. Prior to this study, the G13-RR strain was backcrossed with an SS strain and re-selected with Cry2Ab2 corn leaf powder diet-overlays at a concentration of 15 µg/cm^2 for multiple generations on two separate occasions (Yang et al. 2020). Additionally, a Bt-susceptible *H. zea* strain was obtained from Benzon Research Inc. (Carlisle, PA) and utilized in these assays as a reference. The Benzon strain is susceptible to Cry1Ac, Cry2Ab, and Vip3Aa, having LC_{50} values relatively similar to the SS strain (TX-SS) that was backcrossed with the G13-RR strain (Kerns et al. 2019).

Similar to Kaur et al. 2019, dilutions of the purified Bt proteins were overlaid onto meridic diet used to rear *H. zea* (Frontier Scientific Agricultural Services, Newark, DE). A repeater pipette was used to dispense 0.8 ml of *H. zea* diet into 128-well trays (C-D International, Pitman, NJ), after which the diet was allowed to cool and solidify. Bt proteins were suspended in 0.1% Triton-X100 and dispensed over the surface of the

diet and allowed to air dry. Overlay concentrations for Cry1Ac and Cry2Aa were 0, 0.01, 0.0316, 0.1, 0.316, 1, 3.16 µg/cm2, and concentrations for Vip3Aa39 were 0, 0.0316, 0.1, 0.316, 1, 3.16, 10 µg/cm2. Each well received a volume of 50 µl of the Bt overlay solution. One neonate was placed in each well and vented lids (C-D International, Pitman, NJ) were used to cover the wells. Each treatment consisted of 16 larvae and was replicated four times. The trays were placed in an environmental chamber for seven days at 26 ± 1°C, 50% RH, and a 16:8 (L:D) h photoperiod. Larval mortality was measured based on the number of dead larvae plus larvae that were severely stunted. Larvae were considered severely stunted if they had not molted past the second instar and weighed less than <1 mg.

Bioassays Using Cotton Plant Tissues

With only the Benzon strain, cotton leaf tissue was used to assess the toxicity of the suites of proteins that are expressed in Bollgard II or WideStrike 3 cotton. Assay procedures were identical to those above with the following exceptions. Rather than using purified Bt proteins, Bollgard II or WideStrike 3 leaf powder was suspended in 0.1% Trition-X100 and diluted so that the surface of each well would receive concentrations of 0.01, 0.0316, 0.1, 0.316, 1, or 3.16 mg/cm^2 of Bt cotton leaf powder. Non-Bt leaf powder was also added to each dilution as needed so that all doses had an equal amount of leaf powder. Additionally, 64 wells received a concentration of 3.16 mg/cm^2 of non-Bt leaf powder as a check treatment. A repeater pipette was used to dispense 200 µl of cotton leaf powder solution into each well to achieve uniform coverage of the entire surface of the diet.

Using identical assay methods, powdered leaf, square, boll, white flower, and pink flower tissues were used to determine how different plant parts would affect assay results. Both the susceptible (Benzon) and resistant (G13-RR) *H. zea* strains were assayed. However, only one overlay dose was tested based on the approximate LC_{70} observed in the Bollgard II leaf-powder assay with the Benzon strain. Each well received a concentration of 0.58 mg/cm^2 of Bollgard II or WideStrike 3 plant tissue. The corresponding non-Bt plant powder was added to each Bt plant powder to match the total amount of tissue used in the previous leaf tissue assays (= 3.16 mg/cm^2). Non-Bt plant tissue overlays were also included as a check.

Analysis

The larval mortality in purified protein and leaf tissue assays was calculated by dividing the number of dead or severely stunted larvae by the total larvae that were assayed in each replicate. Mortality was corrected using Abbott's formula (Abbott 1925), and a probit analysis was performed to obtain LC_{50} values and 95% confidence limits (SAS ver. 9.4, SAS Institute, Cary, NC). In some cases, a probit analysis was not performed due to low mortality and the LC_{50} was considered to be greater than the highest dose tested if it resulted in mortality that was less than 50%. Resistance ratios were calculated by dividing the LC_{50} of the G13-RR strain by the LC_{50} of the susceptible Benzon strain.

Larval mortality in the cotton plant part assays was calculated by dividing the number of dead larvae by the total number of larvae that were assayed. For this assay, three different standards for larval mortality were used to evaluate how it affected assay

results. Mortality standards included larvae that were truly dead (Dead), dead or severely stunted and still in the L1 stage (Dead+L1), or dead plus severely stunted and in the L1 and L2 stage (Dead+L1+L2). Larval mortality was only corrected with Abbott's formula when the Dead+L1 mortality standard was used due to excessively high check mortality (>20%) or low corrected mortality in the other mortality standards. Variables that resulted in negative mortality after mortality corrections were excluded from analyses. These data were analyzed using GLIMMIX procedures (α=0.05, SAS ver. 9.4, SAS Institute, Cary, NC). Fixed effects included in the model were strain, trait, plant structure type, and all possible interactions. Replication was included as a random effect. The weights of larvae that fed on non-Bt cotton overlays were analyzed using GLIMMIX procedures with strain, structure type, and the two-way interaction included as fixed effects and replication included as a random variable. Percent growth inhibition for each replicate was calculated using the following formula, [(mean weight larvae in check – mean weight larvae in treatment)/ mean weight larvae in check)*100]. Percent growth inhibition was analyzed using GLIMMIX procedures with the same fixed effects and random variables that were included in the mortality analysis. A Bonferroni post-hoc procedure was used to prevent Type I error on all GLIMMIX analyses.

Results

Purified Protein and Leaf Powder Diet-overlay Assays

LC_{50} values and confidence limits, slopes, resistance ratios, and statistical fit parameters from the probit analyses of the Benzon and G13-RR strains are found in Table 7. The LC_{50} value for the Benzon strain when fed diet overlaid with Cry1Ac was 0.116 µg/cm^2. Comparatively, the G13-RR strain had less than 50% mortality when

exposed to a concentration of 10 µg/cm^2, thus indicating a resistance ratio of >86.2 fold. The Benzon strain had an LC_{50} of 0.058 µg/cm^2 when fed diet overlaid with Cry2Aa, compared with a dose of >3.16 µg/cm^2 needed to kill 50% of the G13-RR strain, indicating a resistance ratio of >54.5 fold. The Vip3Aa39 assays indicated an LC_{50} of 0.51 µg/cm^2 for the Benzon strain, and the G13-RR strain had an LC_{50} value < 0.1 µg/cm^2. Benzon larvae fed diet overlaid with Bollgard II cotton leaf powder had an LC_{50} of 0.208 versus an LC_{50} of 0.955 for assays with the WideStrike 3 leaf powder (Table 7). The dose-response mortality curves for these assays are shown in Figure 2.

Plant Part Assay – Larval Mortality

Figure 3 presents the percent larval mortality (uncorrected) when various larval mortality standards were used. Both tested strains had >60% mean mortality on non-Bt bolls when the Dead+L1+L2 larval mortality standard was used. The G13-RR strain also had >50% mean mortality when fed non-Bt leaves and the Benzon strain had >20% mean mortality on non-Bt squares. Changing the standard for larval mortality to Dead+L1 substantially lowered larval mortality on non-Bt structures. However, larval mortality still remained >20% on non-Bt bolls. Larval mortality on all non-Bt structures was less than 20% for both strains when only dead larvae were included in mortality calculations. Only flowers had <20% mortality for both strains across all three mortality standards (Figure 3). All main effects and two-way interactions were significant regardless of which mortality standard was used. However, the three-way interaction was only significant when the Dead+L1+L2 mortality standard was used (Table 8).

After larval mortality (Dead+L1) was corrected, all the main effects of Bt trait, *H. zea* strain, and plant tissue and their two-way interactions were significant (Table 8). Overall, the Benzon strain had over twice as much mortality as the G13-RR strain, and Bollgard II tissues had higher mortality than tissues from WideStrike 3. White flowers caused the highest mortality regardless of trait. The Benzon strain had over twice as much mortality on Bollgard II diet compared with WideStrike 3 diets. In contrast, no difference in mortality was observed when G13-RR larvae were fed Bollgard II or WideStrike 3 tissues. The corrected larval mortalities when each strain was fed different tissues from Bollgard II or WideStrike 3 cotton are shown in Figure 4. The differences in mortality between the susceptible (Benzon) and resistant (G13-RR) *H. zea* strains were greatest for square and white flower tissues (Table 9, Fig. 4).

Plant Part Assays – Larval Weights

Mean larval weights showed that larvae were substantially stunted when they were placed on non-Bt leaf or boll overlays, and there was an interaction between *H. zea* strain and tissue type (Table 10). The Benzon strain weighed more than twice as much on non-Bt white flowers when compared with non-Bt squares (Fig. 5). In contrast, there was no difference in the mean larval weights of the G13-RR strain when fed tissues from non-Bt squares, white flowers, or pink flowers (Table 10, Fig. 5). Both strains experienced > 50% growth inhibition across both Bt traits and plant tissue types (Table 11, Fig. 6). The G13-RR strain was notably less inhibited by leaves compared with flowers and squares. Similarly, the Benzon strain experienced less growth inhibition on WideStrike 3 leaves compared with Bollgard II (Table 11, Fig. 6).

Discussion

Bt protein diet-overlay bioassays indicated that the G13-RR strain was >50-fold resistant to Cry1A and Cry2A compared with the susceptible Benzon strain, whereas the resistant strain was more susceptible to Vip3Aa. This phenomenon has been observed in similar bioassays conducted on bollworm populations collected from the field (Yang et al. 2017, Yang et al. 2018, Kerns et al. 2019). There may be a trade-off associated with resistance to one or more of the Cry proteins that results in increased susceptibility to Vip3Aa, thus mitigating resistance to multiple Bt proteins expressed in pyramided Bt cotton. However, Kerns et al. (2019) observed that two laboratory susceptible *H. zea* strains reared on meridic diet for multiple generations were more tolerant to Vip3Aa than strains that were reared on meridic diet for only one or two generations. Thus, because the Benzon strain has a longer history of rearing on meridic diet compared with the G13-RR strain, it similarly may have become more tolerant to higher concentrations of Vip3Aa, meaning that this is unrelated to having resistance to other Bt toxins.

The diet-overlay bioassays with cotton leaf tissue suggest that the Benzon strain was more susceptible to Bollgard II (Cry1Ac + Cry2Ab) compared with WideStrike 3 (Cry1Ac +Cry1F + Cry2Ab). This was unexpected considering that WideStrike 3 cotton generally performed better than Bollgard II cotton in field trials conducted across the southern United States (Kerns et al. 2018). However, field populations have developed resistance to the Bt proteins expressed in Bollgard II (Yang et al. 2018), and a laboratory susceptible strain may perform differently given the possible Vip3Aa susceptibility tradeoffs associated with Cry1A and Cry2A resistance that were

previously discussed. Additionally, WideStrike 3 and Bollgard II cotton result from different transgenic insertions that likely confer differential expression of Bt proteins. Expression levels of Bt proteins can also vary among different plant structures, temporally as the plant ages, and among varieties (Adamczyk et al. 2001, Kranthi et al. 2005, Sivasupramaniam et al. 2014). Consequently, the quantity of Bt toxins expressed in the leaf tissue of the WideStrike 3 and Bollgard II varieties used in our assays is unknown. Alternatively, larvae may have exhibited a greater aversion to WideStrike 3 leaf tissue than Bollgard II leaf tissue, thus larvae consumed more Bollgard II leaf tissue and, consequently, consumed more Bt proteins. This is likely, considering that larvae were observed to exhibit a greater aversion to leaf tissue than other plant tissue types that were assessed.

The results from the Bt cotton powder diet-overlay assays suggest that Bollgard II was generally more toxic to *H. zea* regardless of the type of plant tissue that was used. This is consistent with the results of the diet-overlay assays using leaf tissue that were done with the Benzon strain. The three different mortality standards that were used to evaluate the cotton plant tissue diet-overlays have all been used in Bt protein diet-overlay assays (Kaur et al. 2019, Reisig et. al 2018, Yang et. al 2018), but this study highlights the importance of considering the mortality standard that is used when evaluating Bt resistance to ensure that populations are properly characterized. The level of perceived resistance in these assays varied substantially depending on which mortality standard was used. When only dead larvae were used to classify mortality, white flowers from Bt cotton caused relatively high mortality with excellent statistical separation in mortality between the susceptible (Benzon) and resistant (G13-RR) strains

of *H. zea*. Using other tissues did not as clearly demonstrate differences in mortality between these two strains (Table 9, Fig. 3). In contrast, white flowers from Bt cotton caused mortality more comparable to other tissue types, with less separation between *H. zea* strains, when dead larvae + 1st and 2nd instars were used to define mortality.

High mortality and developmental inhibition was observed when bollworms were placed on non-Bt boll tissue in diet-overlays assays (Figs. 3 and 5). Indeed, lower mortality was observed with boll tissue from WideStrike 3 than with non-Bt boll tissue. *H. zea* larvae have exhibited a preference for bolls in late instars but are more likely to feed on squares in earlier instars (Farrar and Bradley 1985), thus small larvae may be less tolerant of secondary metabolites associated with boll tissue and actively attempt to avoid feeding on the tissue. Furthermore, Bt proteins have been known to have anti-feedant properties (Whalon and Wingerd 2003), thus the combined inherent toxicity of boll tissue paired with the cessation of feeding in response to secondary metabolites and Bt proteins may explain the reduced mortality in WideStrike 3 boll tissue. Similar to bolls, the development of larvae on non-Bt cotton leaf tissue was inhibited (Fig. 5). Leaves of Bt cotton collected from near the terminal have been reported to have high concentrations of Cry1 (i.e., Cry1Ac and Cry1F) and moderate concentrations of Cry2Ab relative to other cotton structures (Willrich Siebert et al. 2009, Sivasupramaniam et al. 2014). However, *H. zea* larvae assayed using Bt cotton leaf tissue experienced lower mortality compared with larvae using other Bt cotton tissues. Larvae also exhibited notable aversion to leaf tissue when assays were being prepared, and they tried to escape from wells containing leaf tissue more rapidly than wells containing other tissues. Thus, larvae may have consumed less diet and toxin when placed on leaf

tissue, explaining both the growth inhibition and the low mortality observed in the assay. This may further explain why Bollgard II tissues generally appeared more toxic than WideStrike 3 tissues. In a similar way, larvae may have exhibited greater aversion to WideStrike 3 tissue than Bollgard II tissue, thus larvae in WideStrike 3 treatments consumed less toxin than larvae in Bollgard II treatments.

Larval weights were higher when larvae were placed on non-Bt flower and square tissues compared with boll and leaf tissues (Table 10). *H. zea* larvae have exhibited a general preference for flowers, and smaller larvae have a tendency to feed on squares (Farrar and Bradley 1985), and it is logical that neonate larvae might perform relatively well on these tissues. White flowers from Bt cotton tissue consistently caused more mortality relative to other tissues (Fig. 4). Notably, there was a disparity in larval mortality on pink flowers when compared with white flowers. Pink flower tissue is in a state of senescence (Ritchie et al. 2007), and concentrations of Bt proteins may decrease as they deteriorate. Gore et al. (2002) reported that *H. zea* larvae prefer to feed on floral tissue in Bollgard cotton, and that crop consultants were often finding larvae under desiccated flower tissue. This might suggest that these structures may have reduced concentrations of Bt proteins, thus making them less toxic to bollworm. The Benzon strain experienced higher mortality on Bollgard II square tissue than WideStrike 3 square tissue. In contrast, the difference in mortality caused by Bollgard II and WideStrike 3 was less obvious for the G13-RR strain in assays using square tissue. Differences in feeding behavior have been observed between susceptible and resistant strains of *H. zea*. Anilkumar et al. (2009) suggests that Cry1Ac may have inhibited the feeding of a resistant *H. zea* strain less than that of a susceptible strain, thus resulting in

greater consumption of toxin by the resistant strain in an assay. Consequently, the feeding behavior of the Benzon strain may have been more influenced by the presumably variable concentrations of Bt proteins associated with different plant tissues; whereas the G13-RR strain may have been less affected, resulting in more uniform feeding between different tissue types.

Assays with single, purified Bt proteins or using plant tissue that expressed two or three proteins were both able to detect the known resistance in the G13-RR strain. However, this study demonstrates the importance of tissue selection for plant-based bioassays. Variation in nutrition, secondary metabolites, Bt protein concentrations, and insect strain genetics can all influence the physiology and feeding behavior of larvae in a bioassay, and thus, affect the sensitivity of resistance assays. Boll and leaf tissue do not appear to be ideal for use in an assay due to inherent toxicity of the tissue and larval aversion. Pink flowers, although a preferred feeding site for larvae, may not be ideal for Bt resistance assays due to the low mortality of larvae placed on pink flower tissue collected from Bt cotton. White flowers appear to be the most ideal tissue type to use for assays due to the inherently low toxicity in the absence of Bt toxins, apparent lack of aversion, and the ability to consistently distinguish between the susceptible (Benzon) and resistant (G13-RR) strains. Square tissue also appears to be an adequate alternative for use in resistance monitoring assays.

Assays using tissue from white flowers may better predict how a bollworm strain may perform when exposed to the suites of proteins that are expressed by pyramided Bt cotton varieties. Subsequent assays using purified Bt proteins could then be used to identify resistance to single Bt proteins when deemed necessary. However, determining

the relative concentrations of the Bt proteins in the different lyophilized powders might further explain the results of these assays. Furthermore, lyophilized verses fresh-tissue assays could be done to determine if assay results would be congruent.

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Appendix

Table 7. LC_{50} values with 95% confidence limits, slope line, and X^2 goodness of fit for the probit lines from three different Bt proteins and two different Bt cotton leaf powders in feeding assays with a Bt-susceptible and Bt-resistant *Helicoverpa zea* strain.

Overlay	Strain	N[a]	LC_{50} (95% CL)[b]	Slope ± SE	X^2	df	RR[c]
Cry1Ac	Benzon	448	0.116 (0.084, 0.160)	1.79 ± 0.2	38.4	22	1.0
	G13-RR	64	>10				>95.2
Cry2Aa	Benzon	448	0.058 (0.047, 0.072)	2.02 ± 0.19	10.8	22	1.0
	G13-RR	64	>3.16				>54.5
Vip3Aa	Benzon	448	0.58 (0.47, 0.71)	2.19± 0.19	9.58	22	1.0
	G13-RR	448	<0.1				<0
Bollgard II	Benzon	448	0.208 (0.141, 0.313)	1.18 ± 0.13	35.1	22	
WideStrike 3	Benzon	448	0.955 (0.489, 2.648)	0.79 ± 0.13	46.1	22	

[a] Total number of neonate larvae assayed.

[b] Larva mortality was calculated based on the number of larvae that were dead plus the number of larvae that were still in the 2nd instar. LC_{50} values were considered greater than the highest concentration tested if less than 50% mortality was observed when assayed at the highest Bt protein concentration.

[c] Resistance ratios were calculated by dividing the LC_{50} value of a Bt resistant population (G13-RR) by the LC_{50} value the Benzon (susceptible) population.

Table 8. Significance of fixed effects on larval mortality in Bt cotton plant tissue assays with *Helicoverpa zea* when the mortality parameter was dead larvae (DEAD), dead plus first instars (Dead+L1), or dead plus first and second instars (Dead+L1+L2*).*

Table of Fixed Effects For Three Different Larval Mortality Standards				
Mortality Standard	**Effect**	**F- Value**	**Df**	**P-Value**
Dead	Trait	38.00	2, 87	<.0001
	Tissue	19.12	4, 87	<.0001
	Strain	6.96	1, 87	0.0099
	Tissue*Trait	3.33	8, 87	0.0023
	Strain*Tissue	3.95	4, 87	0.0010
	Strain*Trait	5.08	2, 87	0.0288
	Strain*Trait*Tissue	1.56	8, 87	0.1496
Dead+L1	Trait	42.40	2, 87	<.0001
	Tissue	24.19	4, 87	<.0001
	Strain	16.68	1, 87	<.0001
	Tissue*Trait	5.76	8, 87	<.0001
	Strain*Tissue	5.03	4, 87	0.0011
	Strain*Trait	5.47	2, 87	0.0058
	Strain*Trait*Tissue	1.41	8, 87	0.2052
Dead+L1+L2	Trait	156.8	2, 87	<.0001
	Tissue	16.71	4, 87	<.0001
	Strain	7.03	1, 87	0.0095
	Tissue*Trait	15.87	8, 87	<.0001
	Strain*Tissue	7.46	4, 87	<.0001
	Strain*Trait	3.91	2, 87	0.0236
	Strain*Trait*Tissue	3.69	8, 87	0.0010

Table 9. Effect of Bt trait, *Helicoverpa zea* strain, and the type of cotton plant tissue on percent larval mortality in diet overlay assays.

Percent Larval Mortality			
Main Effect	**Treatments**	**% Mortality[a]**	**SEM**
Trait	Bollgard II	24.95a	3.3
	WideStrike 3	13.12b	2.4
Strain	Benzon	28.12a	3.7
	G13-RR	11.38b	2.1
Tissue	Leaves	8.64b	2.2
	Squares	15.41b	3.2
	W. Flowers	54.72a	4.7
	P. Flowers	10.80b	2.4

Type III Tests of Fixed Effects			
Main Effect	**F-Value**	**df**	**P-Value**
Trait	14.83	1, 45	0.0004
Strain	29.58	1, 45	<.0001
Tissue	47.67	3, 45	<.0001
Trait*Strain	4.77	1, 45	0.0341
Trait*Tissue	3.70	3, 45	0.0184
Strain*Tissue	3.22	3, 45	0.0314
Trait*Strain*Tissue	0.11	3, 45	0.9526

[a] Percent mortality is based on the number of larvae that were dead plus the number of larvae that were still in the first instar (Dead+L1). Mortality was corrected using Abbott's formula using non-Bt cotton tissue as a check.

Table 10. The effect of *Helicoverpa zea* strain and the type of non-Bt cotton plant tissue on the mean weight of individual larvae in diet overlay assays.

Mean Larva Weight in Non-Bt Treatments			
Effect	**Treatments**	**Mean Larva Weight (mg)**	
Strain	Benzon	13.23a	
	G13-RR	9.99b	
Tissue	Leaves	3.76c	
	Bolls	1.60c	
	Squares	13.14b	
	W. Flowers	20.82a	
	P. Flowers	18.74a	
Type III Tests of Fixed Effects			
Effect	**F-value**	**df**	**P-Value**
Strain	21.07	1, 27	<.0001
Tissue	120.7	4, 27	<.0001
Strain*Tissue	16.74	4, 27	<.0001

Table 11. Effect of Bt trait, *Helicoverpa zea* strain, and type of cotton plant tissue on the percent growth inhibition of larvae in cotton plant tissue assays.

Percent Growth Inhibition			
Main Effect	**Treatments**	**% Inhibition[a]**	**SEM**
Trait	Bollgard II	92.8a	1.48
	WideStrike 3	86.4b	2.24
Strain	Benzon	93.2a	1.41
	G13-RR	85.7b	2.34
Tissue	Leaves	73.6	4.05
	Squares	90.2	2.27
	W. Flowers	96.1	1.13
	P. Flowers	91.4	2.08

Type III Tests of Fixed Effects			
Main Effect	**F-Value**	**df**	**P-Value**
Trait	10.27	1, 45	0.0025
Strain	13.86	1, 45	0.0005
Tissue	16.31	3, 45	<.0001
Trait*Strain	0.62	1, 45	0.4357
Trait*Tissue	0.92	3, 45	0.4398
Strain*Tissue	2.07	3, 45	0.1174
Trait*Strain*Tissue	1.49	3, 45	0.2287

[a] Percent growth inhibition calculated using ((non-Bt mean larval weight-Bt mean larval weight)/non-Bt mean larval weight)*100.

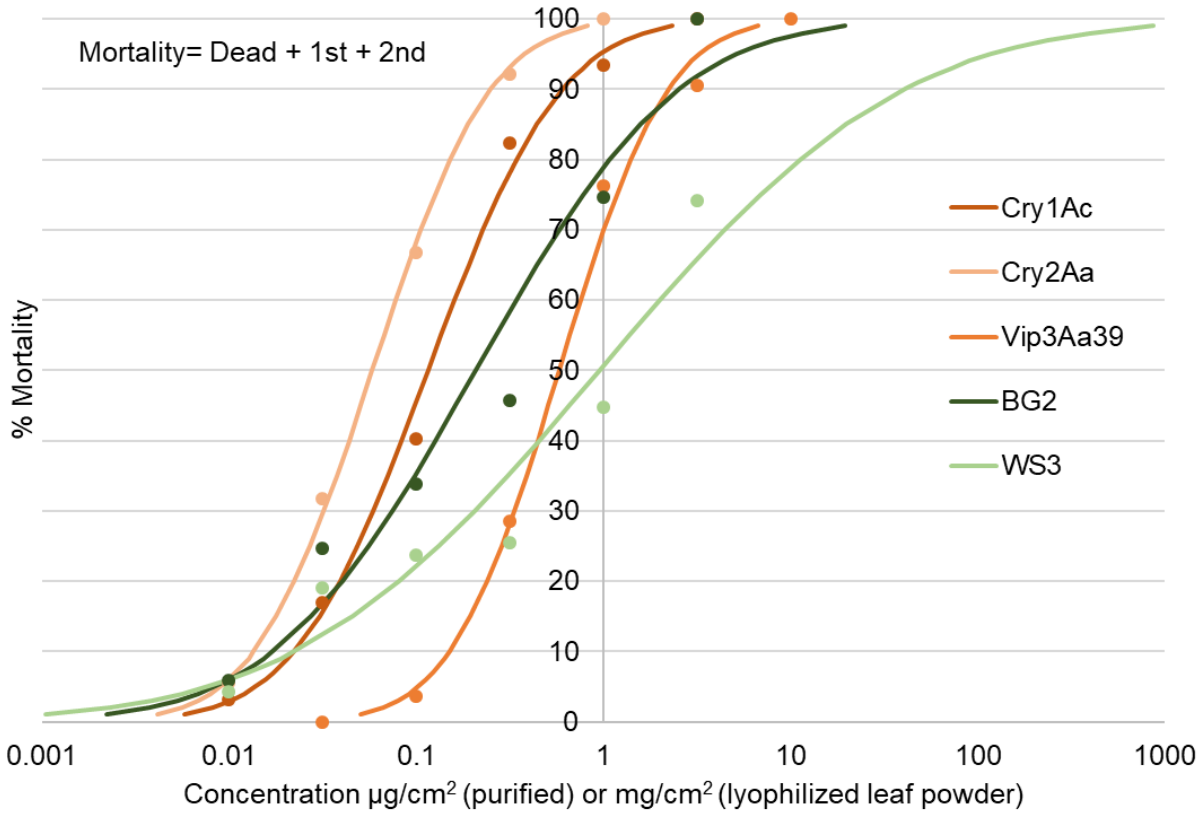

Figure 2. Predicted mortality response of a susceptible strain (Benzon) of *Helicoverpa zea* larvae to three different Bt proteins and to leaf tissue from cotton varieties with different Bt traits. Mortality is based on the number of dead larvae plus larvae that were still in the first or second instar (Dead+L1+L2).

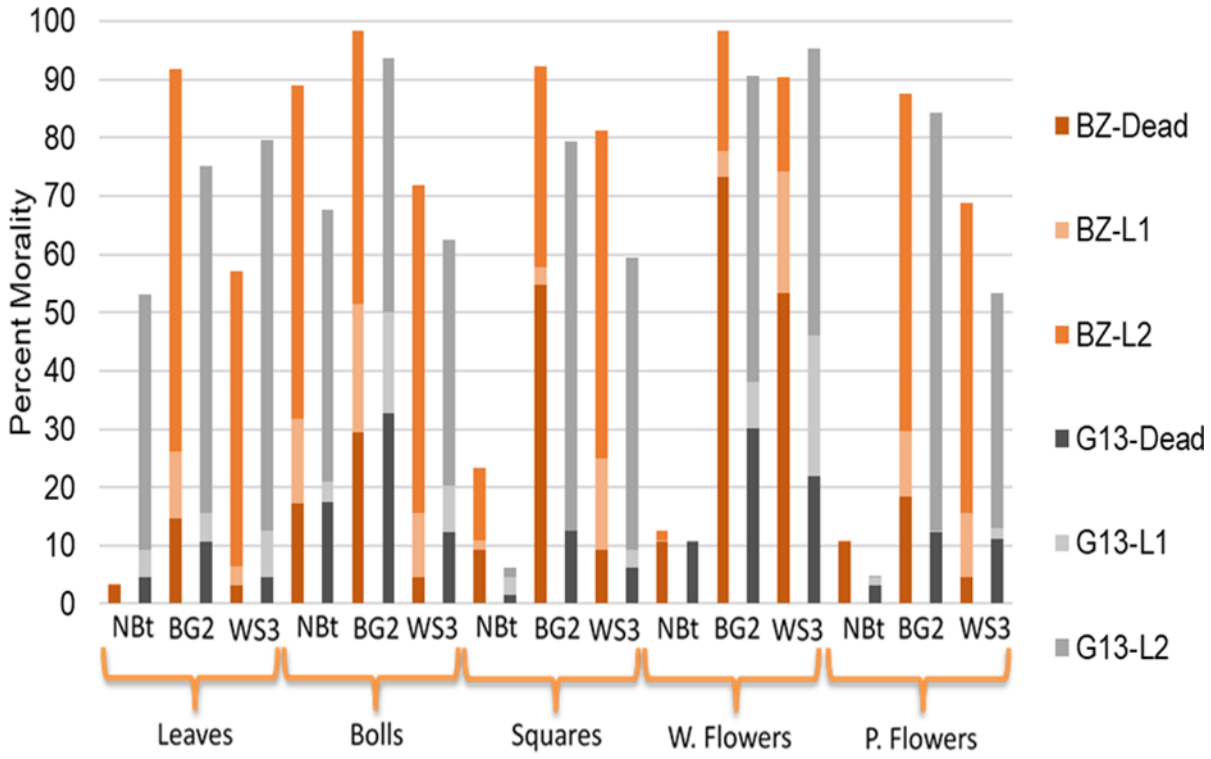

Figure 3. Percent larval mortality using three different mortality parameters for assays using Bt cotton (Bollgard II and WideStrike 3), different types of plant tissue, and a Bt-susceptible (BZ, Benzon) and resistant (G13-RR) strain of *Helicoverpa zea*.

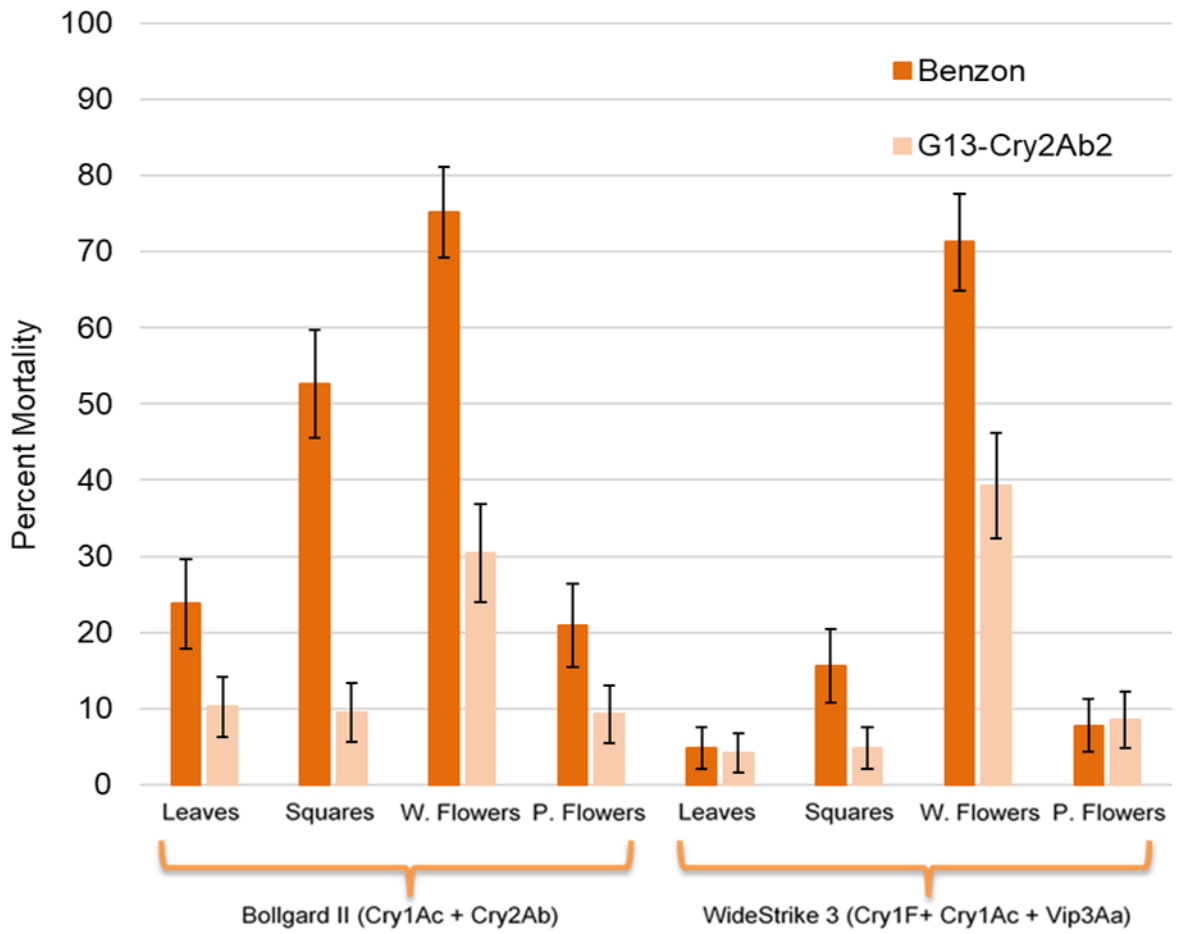

Figure 4. Larval mortality (Dead+L1) after corrections with Abbott's formula based on assays using Bollgard II or WideStrike 3 cotton plant tissues and a Bt-susceptible (Benzon) and resistant (G13-RR) strain of *Helicoverpa zea*.

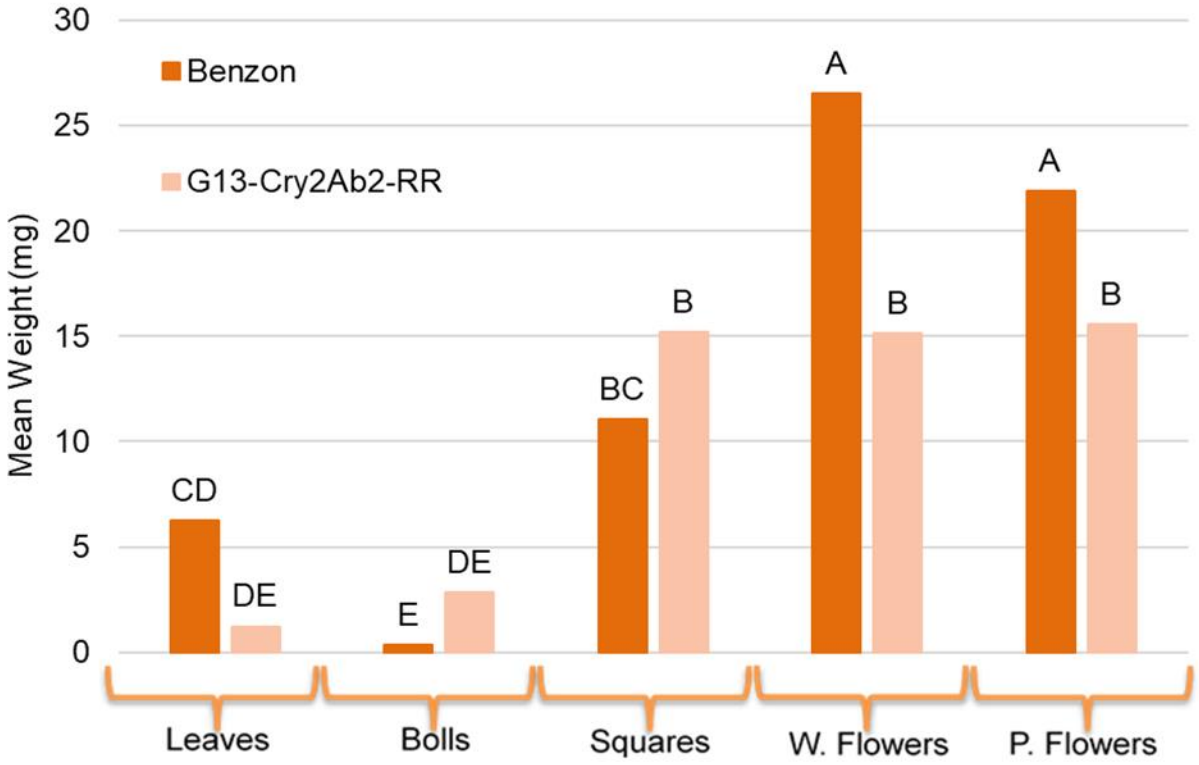

Figure 5. Larval weights of a Bt-susceptible (Benzon) and resistant (G13-RR) strain of *Helicoverpa zea* when fed non-Bt cotton plant tissues in a diet overlay assay (Strain*Tissue: $P < 0.05$, SEM ± 1.1151).

Conclusions

The first objective was to determine if different Bt cotton technologies or the application of an insecticide had a significant effect on the distribution of bollworm larvae and their feeding. This information should help in the development of simplified scouting methodologies that are standardized across non-Bt or Bt technologies, regardless of whether an insecticide was previously applied. The presence of *H. zea* larvae and feeding was most prevalent in the middle portion of the cotton canopy regardless of the Bt traits associated with a cotton variety. Small larvae were found more often in the middle and upper portions of the cotton canopy where flowers were present during the time of sampling. This suggests that adults attracted to the flowers may have oviposited on or near flowers. Larger larvae were found lower in the canopy, likely reflecting downward movement into the canopy as they aged. Although different Bt traits may have affected how quickly larvae moved downward in the cotton canopy, it did not substantially affect the distribution of larvae or their damage. Similarly, insecticide application had modest impact on larval distribution, and injury and effects were more apparent in the upper canopy. Sampling of pink flowers and small bolls from the middle portion of the canopy appears to be the best sampling technique for detecting bollworm infestations and making insecticide treatment decisions. This method would accommodate both larval or injury based treatment thresholds, and it could be used regardless of if or what Bt cotton technologies are used. However, alternative scouting methodologies may be justified for conditions of very high or early onset of bollworm infestations.

The second objective was to determine if bioassays using cotton plant tissues could aid in detecting Bt resistance and reflect how bollworm populations would perform when exposed to Bt cotton under field conditions, and if so, which plant tissues would be the most ideal to use for diet-overlay bioassays. Assays using purified Bt proteins confirmed the resistance of a bollworm strain (G13-RR) to Cry1Ac and Cry2Aa when compared with a laboratory susceptible strain (Benzon). However, the resistant strain was more susceptible to Vip3Aa39 than the susceptible strain. Assays using boll tissue from non-Bt bolls caused larval stunting and high larval mortality. Similarly, *H. zea* larvae experienced a similar stunting of growth in assays with non-Bt leaf tissue and also exhibited an aversion to the leaf tissue. The results suggested that tissue from white flowers were the most ideal for diet-overlay bioassays to detect Bt resistance. Squares may also be an adequate option for bioassays. Pink flowers did not achieve the desired level of toxicity for an adequate assessment of Bt resistance. This may have been due to low concentrations of Bt proteins in pink, senescing flower tissue. Resistance to the Cry proteins was detected using both Bollgard II and WideStrike 3 white flower tissue in diet-overlay assays. Ideally white flower tissue diet-overlays could be used in conjunction with Bt protein diet-overlays to better assess how a resistant population would perform under field conditions when exposed to pyramided Bt toxins in plant tissues.

Nutrition, secondary metabolites, and Bt protein concentration can affect bollworm behavior and survival on Bt cotton. Considering these factors and their interactions can aid in developing improved scouting methodologies and resistance monitoring bioassays that are more field applicable. This will likely become increasingly

important as new Bt technologies are developed and commercialized. Resistance monitoring efforts carried out across the Cotton Belt show that *H. zea* field populations have developed resistance to the Cry proteins expressed in current Bt cotton technologies. However, it is easy to underestimate the collective effect that a suite of Bt proteins may have on various bollworm populations under field conditions. Results from these studies demonstrate that 'older' Bt technologies still provide considerable plant protection, even though the need for supplemental insecticide applications may be increased because of resistance to Cry proteins. The biological and ecological characteristics of *H. zea* challenges the major resistance management strategies that have been implemented to mitigate the development of resistance to Bt corn and cotton. Nonetheless, with the integration of new Bt traits, bollworm resistance to Cry Bt toxins is currently manageable, especially if foliar insecticide alternatives such as applications of chlorantraniliprole remain effective.

Vita

Dawson Kerns was born in Yuma, AZ. From a young age, he had an interest with insects and he first developed an interest in agriculture after working in cotton fields in Lubbock, TX for a summer job when he was 15 years old. In 2018, he obtained a B.S. in Entomology from Texas A&M University. He then pursued a M.S. in Entomology under Dr. Scott D. Stewart at The University of Tennessee in the Department of Entomology and Plant Pathology. He plans to obtain a PhD under Dr. Juan Luis Jurat-Fuentes and Dr. Scott D. Stewart.