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# Susceptibility of Field-Collected Pupations of the Corn Earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) from Three Southern States of the U.S. to Cry1A.105 and Cry2Ab2 Proteins

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**SUSCEPTIBILITY OF FIELD-COLLECTED PUPATIONS OF THE  
CORN EARWORM, HELICOVERPA ZEA (BODDIE) (LEPIDOPTERA:  
NOCTUIDAE) FROM THREE SOUTHERN STATES OF THE U.S. TO  
CRY1A.105 AND CRY2AB2 PROTEINS**

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

in

The Department of Entomology

by  
Gagandeep Kaur  
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## ABSTRACT

The corn earworm (CEW), *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), is a major target pest of pyramided Bt corn and Bt cotton in the U.S. In 2016 and 2017, notable corn ear damage and larval survival of CEW were observed on pyramided Cry1A.105/Cry2Ab2 corn in some fields in northeast Louisiana. The objectives of this study were 1) to determine if the ear damage and larval survival observed in the area were due to resistance development to the Bt proteins in the plants, and 2) if resistance had occurred, to determine the approximate distributions of the resistance in the southern region of the U.S. To accomplish the proposed objectives, 12 populations of CEW were collected from Bt and non-Bt corn plants in multiple locations in Louisiana, Georgia, and Florida. Diet-overlay bioassays were conducted to examine the susceptibility of the progeny produced from the field-collected populations to Cry1A.105 and Cry2Ab2. Results of the bioassays showed that the median lethal concentrations ( $LC_{50}$ s) of Cry1A.105 and Cry2Ab2 for the populations collected from the areas with control problem occurrence were as much as >909-fold and >25-fold greater than that of a known Bt-susceptible strain, respectively. The results documented that the observed field control problems of Cry1A.105/Cry2Ab2 corn in northeast Louisiana was due to resistance development of the insect to the Bt proteins in the plants. This is the first documentation of field resistance to Bt corn in any target insect species in the U.S. mid-south region. However, susceptibility levels to Cry1A.105 and Cry2Ab2 varied greatly among the CEW populations collected from the three states, suggesting a mosaic distribution of the resistance in the region. Several factors could have contributed to the rapid development of the resistance to Cry1A.105/Cry2Ab2 corn plants in the insect. The documentation of the field resistance to Cry1A.105/Cry2Ab2

corn in CEW should have important implication for development of effective resistance management strategies for the sustainable use of Bt crop technology in the region.



# **1. INTRODUCTION**

## **1.1. Corn in the U.S.**

Field corn (*Zea mays*) is a major crop in the U.S. It has a significant role in the U.S. economy. Total corn planted area in the U.S. in 2017 was 90.2 million acres and production was 14.6 billion bushels (NASS, 2018). In 2017, corn crop was harvested from 82.7 million acres with a crop value of \$47.5 billion (NASS, 2018). In Louisiana, corn is also a major field crop as it was planted on 500,000 acres with a production of 90,160 thousand bushels from 490 thousand harvested areas in 2017 (NASS, 2018).

## **1.2. Uses of corn**

Corn is one of the important food and feed crop in the world. Earlier, corn was used mainly for these two purposes, but with time it's uses have expanded such as it has been used in fuel production and brewing industry. Corn is a very nutritious crop, rich with nutrients, fibres, proteins, carbohydrates, and vitamins. Some of the corn products are corn syrup, corn oil, corn starch, popcorn, grilled corn, corn flakes, etc. Corn has constituted a major part of American life. For examples, in 2017 alone, approximately 6,434 thousand acres of corn were harvested for silage and 35,835 thousand bushels of corn were used for beverage alcohol production in the U.S. (NASS, 2018). In addition, about 5,493,881 thousand bushels of corn were used in fuel industry in 2017 alone (NASS, 2018).

## **1.3. Major insect pests of corn**

There are many insect pests of corn, which feed on almost every part of corn plants. These insect pests are broadly divided into different categories based upon their feeding habits such as seed, root, and lower stem feeders, stalk borers, leaf feeders, and ear feeders (Radcliffe and Hutchison, 1999). The common seed feeders include the seed corn maggots, seed corn beetles, wireworms,

etc. They feed on corn seeds in soil, which results in no emergence of corn plant. White grubs, rootworms including the western corn rootworm (*Diabrotica virgifera virgifera*), southern corn rootworm (*Diabrotica undecimpunctata howardi*), and wireworms feed on corn roots, while chinch bugs (*Blissus leucopterus leucopterus*), black cutworms (*Agrotis ipsilon*) are lower stem feeders. Stalk borers are the European corn borer (*Ostrinia nubilalis*), southwestern corn borer (*Diatraea grandiosella*), sugarcane borer (*Diatraea saccharalis*), southern cornstalk borer (*Diatraea crambidoides*) and lesser cornstalk borer (*Elasmopalpus lignosellus*). These borers feed on corn stalk and affect xylem-phloem transport in plants. They can also feed on other plant parts such as leaves and ears. The third category is leaf feeders include a complex of aphids, thrips, mites, armyworm, grasshoppers, cutworms, and stink bugs. These insects feed on corn leaves, which are the primary source of photosynthesis of the plants. Some of these pests sap or eat some portion of leaves which results in low chlorophyll content, consequently less photosynthesis and reduced plant growth. The last is ear feeders, these insects feed on the most economic part of the corn such as corn ears. They are corn earworm (CEW) (*Helicoverpa zea*), western bean cutworm (*Striacosta albicosta*), fall armyworm (*Spodoptera frugiperda*) and cutworms (*Agrotis segetum*). They feed on corn ear and make it unsuitable for market. Above mentioned are some of the important insect pests of corn in the U.S., but there are many more, although damage caused by them may be not common as those mentioned above.

#### **1.4. Corn earworm (CEW)- biology, damage, and distribution**

CEW is a major agricultural pest of corn. It is an insect species of family Noctuidae. It is a polyphagous pest; its larvae feed on many crops. It is also known with other names as cotton bollworm, tomato fruit worm. The newly produced eggs are pale yellow and are laid on leaf hairs and silks (Neunzig, 1964). CEW larvae have five to six instars; they mostly feed on reproductive

parts of the plants. They pupate below the soil surface. The crucial factors for pupae are temperature and soil moisture; low temperature and high soil moisture lead to increase mortality of pupae (Barber, 1937; Ditman et al., 1940). Adults are nocturnal and usually hide in vegetation during day (Kogan, 1978). Adults can live up to 12 to 16 days. CEW also feeds on many other crops, such as cotton, tomato, potato, sorghum, etc., but corn, cotton, and sorghum are usually its most favourite hosts. Damage to corn is mainly caused by larvae feeding on ear kernels. In the southern region of the U.S., where cotton is also planted, CEW, after corn senescence, moves to other hosts, notably cotton, grain sorghum and soybean, for 2-3 additional generations. Because CEW is also a major target pest of Bt cotton in the south region of the U.S., it presents a significant challenge for resistance management. There is the potential for multiple exposures to Bt proteins across generations in both Bt corn and Bt cotton (US EPA, 2001).

### **1.5. Management of corn earworm**

CEW larvae damage corn mainly by ear feeding. Corn ear is the edible part of the corn, which makes CEW management difficult. Unlike other insects, which feed on leaves, for example the fall armyworm, which can be controlled by spraying insecticides on leaves, corn ear is the economic part and more cares need to be taken when insecticides are used to control CEW. Methods of CEW management include – sampling, cultural practices, insecticide use, biological control, and host plant resistance. CEW adults can be sampled with pheromone and black light traps. Pheromone traps are effective for sampling females only, whereas black light trap is effective for both males and females. A very common cultural practice is to plant trap crops. Trap cropping is planting of crop which has less cash value and more attractive to the pest in or around the high value cash crop to lure insects (Shelton and Badenes-Perez, 2006). Corn at silking stage is the most attractive to the ovipositing females of CEW (Johnson et al., 1975). It

can be used to lure the insects from main crops, but disadvantage of this method is that the attractive period cannot be maintained for prolonged time. In addition, planting time is also an important cultural practice that can be used to reduce CEW populations in the U.S. Early planting (e. g. before April 15 in Louisiana) is usually very effective to reduce CEW occurrences and insecticide applications to control CEW are usually not necessary for the early planted corn in Louisiana. Tillage is also effective against CEW because it pupates in soil and overwinters in the pupal stage. Insecticides are commonly used to control CEW larvae. However, because CEW larvae reside inside the corn ear, it is difficult to kill the larvae with insecticide. Insecticide application decision making usually depends upon the number of adults captured in traps (Flood et al., 2005). Another safe method is biological control- application of *Trichoderma* eggs can control CEW larvae (Oatman, 1966). However, this method usually is not used on large scale, but it is feasible for small home gardens. Disadvantage of biological control is that CEW larvae feed on corn ear and then pupate in soil, and thus some damage has occurred before the pest is controlled. Another effective method against CEW is the use of host plant resistance. Resistant host can depend on different factors such as physical- husk tightness, chemical- myosin content, etc. The most successful biological control for CEW management is the use of *Bacillus thuringiensis* (Bt). The advantages of biological control include environment friendly, no or less use of insecticides, no harm to mammals, natural enemies, birds, predators etc.

#### **1.6. Bt and bioengineered corn- Bt corn**

Bt is a rod-shaped soil bacterium that produces specific crystalline endotoxin (Cry) during the reproductive stages and vegetative insecticidal proteins (Vip) during the vegetative growth stages. Both Cry and Vip are toxic to specific insect species (Gasser and Fraley, 1989; Vaeck et al., 1989). Bt is an endospore-forming bacterium that produces a protein crystal within the

cytoplasm of sporulating cells (Schnepf et al., 1998). The mode of action of Bt to kill insect is still not fully understood. To kill insect, Bt protein is first ingested by the insect. After ingestion into the insects, it is converted to the active form by enzyme proteinases present in the midgut. Then, it disintegrates the midgut membrane by binding to the midgut receptors and forms the pore in the membrane that ultimately causes the cell swelling and lysis, and finally causes the cell death to kill the insect.

### **1.7. Bt resistance**

Transgenic plants possessing Bt genes provide a safe and effective method for controlling insect pests. However, the rapid and large scale adoption of Bt crops has allowed the insects to evolve resistance to the Bt proteins. Many cases of insect resistance to Bt crops have been reported. The first ever laboratory-selected resistance to *Bt* was reported in the house fly (*Musca domestica*) to a Bt formulation (Harvey and Howell, 1965). Later, a high level laboratory-selected Bt resistance was reported in the Indian meal moth (*Plodia interpunctella*) (McGaughey and William, 1985). These works were all under laboratory conditions. Field resistance to Bt microbial insecticides (Dipel) was first reported in the diamondback moth (*Plutella xylostella*), a major pest of vegetables (Tabashnik et al., 1990). Later, the cabbage looper (*Trichoplusia ni*) was also developed resistance to Bt insecticide application in the greenhouse (Janmaat and Myers, 2003). Field resistance to Bt has been documented in field transgenic crops which are our major area of concern. The major ones are - resistance in the African stem borer (*Busseola fusca*) to Cry1Ab corn in 2005 in South Africa (van Rensburg, 2007), resistance in the pink bollworm (*Pectinophora gossypiella*) to Cry1Ac cotton in India (Dhurua and Gujar, 2011), western corn rootworm to Cry3Bb1 corn in 2011 in USA (Gassmann et al., 2011), and the fall armyworm to

Cry1F corn in Puerto Rico (Storer et al., 2010), Brazil (Farias et al., 2014), the southeast region of the mainland U.S. (Huang et al., 2014), and recent in Argentina (Chandrasena et al., 2018).

Based on the above mentioned mode of action, insects may develop resistance to Bt toxins by mainly two ways, either by not converting Bt toxin to the active form by proteinases or by not allowing it to bind the midgut receptor. Lot of work has been done and is still going on to find the Bt resistance mechanisms. Some researchers concluded that cadherin, aminopeptidase, and alkaline phosphatase are among the common midgut receptors for Bt binding (Yang et al., 2011). Numerous researchers are working to determine the physiological and molecular mechanisms of Bt resistance in insects.

## **1.8. Resistance management**

In the U.S. and several other countries, two insect resistance management (IRM) strategies have been adopted to maintain the sustainability of Bt crops, which are a ‘high-dose/refuge’ strategy and a gene-pyramiding strategy, along with resistance monitoring for all target pests of Bt crops (Matten et al., 2012).

### **1.8.1. Gene stacking and pyramiding**

Gene-stacking is different from gene-pyramiding. In gene-stacking, more than one Bt genes are transferred into the plants for different purposes or controlling different insect-pests, while in gene-pyramiding, two or more transferred Bt genes are against the same target species (Huang, 2015). An example of gene stacking is YieldGard Plus, which has two Bt genes, Cry1Ab for controlling corn borers and Cry3Bb1 for controlling corn rootworms. A good example of gene pyramiding is Bollgard II cotton, which contains Cry1Ac and Cry2Ab2, both for controlling moth pests such as budworm and bollworms. A key requirement for the success of the gene

pyramiding is that no cross-resistance exists among the Bt pyramided proteins in the plants. Otherwise, insects can easily develop cross-resistance (Manyangarirwa et al., 2006).

### **1.8.2. 'High dose/refuge' IRM strategy**

Another IRM strategy for planting Bt corn is the 'high dose/refuge' strategy. It is basically planting the high dose Bt plants in one portion of the field and non-Bt plants in the remaining field (Huang et al., 2011). This strategy is used to maintain the resistance (R) allele frequencies at a low level. This strategy works as the refuge (non-Bt plants) hosts the local population of insect without Bt resistant alleles that can mate with the population from Bt crop having two resistant alleles. Thus, heterozygous (RS) population will be produced in their offspring, which can be killed by the high dose Bt corn. High dose of Bt proteins is suggested as the '25 times more than the concentration needed to kill the susceptible (SS) larvae', so that both SS and RS (heterozygous) can be killed by the high dose (US EPA, 1998). However, the level of high dose is not same for all insects and crops. It is different for the crops and even for the same crop with different plant stages. Other important assumptions for this strategy include that the resistance is functionally recessive, or at least partially recessive; initial resistance allele frequency is very low ( $<0.01$ ) and there is random mating between susceptible and resistant insects. If these assumptions are not met, resistance could develop rapidly. Field resistance to Bt crops has occurred in several target pests as mentioned above. For all these cases, the reasons are assumed that the three assumptions of the 'high dose/refuge' strategy have not been met (Huang et al., 2011).

### **1.8.3. Bt resistance monitoring**

In addition, resistance monitoring should be done to measure the resistance allele frequency before it causes field control problems. It is usually hard to detect resistance alleles when their

frequencies are very low in the field. For this reason, resistance monitoring at low resistance allele frequencies is often costly. On the other hand, resistance monitoring programs for Bt crops should be sensitive enough to measure the resistant allele frequency so proactive actions can be employed before field control problems occur (Huang, 2006). There are several methods for resistance monitoring, such as 1) reports obtained from growers about field control problem, 2) dose-response bioassay, 3) diagnostic/discriminating dose bioassay, 4) F2 screen, 5) screening against known laboratory resistant insects (F1 screen), 6) sentinel pots, field survey plus laboratory, and 7) DNA marker method (Huang, 2006).

Information based on growth reports may be too late to employ any proactive actions to manage the resistance because when growers find the control problem, the insect already becomes resistant. For dose-response bioassay, insects are collected from field and then they are reared for one or more generations. Laboratory bioassay is done by using different Bt concentrations to determine the lethal doses, which can be used to compare to the value of reference (susceptible) populations or historical data. This method is very useful in validating resistance. However, it is also not sensitive to detect rare resistance alleles in field insect populations. Relative to the dose-response bioassay, discriminating dose bioassay is more powerful. In discriminating dose bioassay only one or two discriminating doses are used and survival is compared at the discriminating doses. In resistance monitoring, probably, no single method can provide accurate information about insect resistance. However, several researchers (Andow and Alstad, 1999; Huang, 2006) have pointed out this method is still not sensitive enough to detect rare resistance alleles in the field.

For F1 screen, larvae or pupae are collected from the field, they are reared in the laboratory, and then they are paired with the lab RR insects to develop F1 generation. This F1 generation is



used for resistance screening. This method is very powerful, but a known resistant strains must be available for the crosses and it can detect only the resistance allele that laboratory strain is present (Yue et al., 2008). The F2 screen is an effective method to measure the resistance allele frequency whether the resistance is dominant or recessive. In F2 screen, mated females or larvae of the insect species interested in are collected from field. Larvae collected from field are reared in the laboratory to adults and single-pairing is used to establish isoline families. F1 adults from field-collected mated females or single-pairings in the laboratory are sib-mated within each isoline family to produce F2 progeny. Progeny survival of each F2 isoline family is screened for Bt resistance at a diagnostic dose (Andow and Alstad, 1999) or using Bt plant tissue (Huang et al., 2007a). Theoretically, 6.25% of the F2 progeny should be homozygous for the resistance and the homozygous resistant individuals should survive at the diagnostic dose or feeding on Bt plant tissues. Several studies have shown that this method is effective to detect rare resistance alleles in field populations, even when the resistance is recessive (Huang et al. 2007). However, the costs of the F2 screen can be a big problem because it requires to rear each family of hundreds of isoline families in the lab for longer than one generation (Yue et al., 2008; Huang et al., 2012).

The sentinel plot method includes planting attractive Bt and non-Bt plants in sentinel plots and then observe the plant damage and insect survival on the plants. Another simple method is the use of field surveys plus laboratory bioassays. In this method, live larvae are collected from Bt plants and continue to be reared in the lab and offspring are screened on Bt plants or diet containing Bt toxin. This method has been used to measure the Bt resistant allele frequency of the pink bollworm (Tabashnik et al., 2000). Probably, the most efficient method to detect Bt resistance alleles is the use of the associated DNA markers. In DNA- screening, cloning and sequencing of the genomic region of mutation for resistance are done and DNA makers

associated with the resistance are then identified by comparing the DNA sequences of resistant and susceptible insects. This method is very efficient because, theoretically, it can identify the resistance in both homozygous and heterozygous resistant individuals, as well as, using live and dead insect body at all the insect growth stages. An example of the use of DNA screening was to detect Cry1Ac resistance in the pink bollworm. The DNA marker screen showed that the resistance allele frequency is rare for Bt cotton in the pink bollworm populations in Arizona (Tabashnik et al., 2000).

### **1.9. Objectives**

Since 1999, Bt corn has been successfully used for managing a complex of caterpillar pests in Louisiana (LA). Both Bt and non-Bt growers in LA have gained considerable benefits from the successful planting of Bt corn with an estimate of a net-return of over \$20 million annually (F. Huang, unpublished data). However, such benefits could be vanished if resistance to Bt crops in insect pests occurs. To ensure the long-term success of Bt corn, scientists from Louisiana State University Agricultural Center (LSU AgCenter) have implemented a resistance monitoring program since 2004. Based on the data collected from the 2017 monitoring, field control problem of some commonly planted Bt corn products in the sentinel fields against CEW was observed. These sentinel fields were planted later than the normal planting date with Bt and non-Bt corn plants for attracting insect pests for monitoring possible resistance development. During the 2017 crop season, sentinel plots were planted in four LSU AgCenter's research stations: The Northeast Research Station in St. Joseph, Macon Ridge Research Station in Winnsboro, Dean Lee Research Station in Alexandria, and Central Research Station in Baton Rouge. The 2017 monitoring showed that >90% ears of corn plants containing the Genuity®SmartStax® trait were

significantly damaged by CEW and >50% ears contained large live CEW larvae in the sentinel plots at the Macon Ridge Research Station in Franklin Parish in

Table 1.1 Percentage and area of corn ears damaged by corn earworm in northeast Louisiana in 2017

Source of ears sampled	Location	Percentage of ear damaged	Kernel damage (cm <sup>2</sup> /area)
100% Bt	Franklin, LA	100	10.1
SMT Bt plant in 90:10% RIB	Franklin, LA	98	8.42
SMT Bt plant in 80:20% RIB	Franklin, LA	98	11.68
100% non- Bt	Tensas, LA	100	11.36

northeast Louisiana. The number of live larvae and ear damage levels were not much different compared to the non-Bt corn plants in the sentinel plots. SmartStax Bt corn contains three Bt toxins (Cry1F, Cry1A.105, and Cry2Ab2) for controlling caterpillar pests including CEW. Corn containing Cry1A.105, Cry2Ab2 and/or Cry1F Bt proteins is the most widely planted Bt corn products in LA and other states of the U.S. The major objective of this study was to determine if the plant damage and larval survival of CEW on the transgenic Bt corn plants observed in the field in northeast Louisiana was due to resistance development to the Bt proteins in the plants. In addition, laboratory bioassays were also conducted for several CEW populations collected from other areas in Louisiana, Florida, and Georgia to determine if field resistance had occurred in other areas in the region.

## **2. MATERIALS AND METHODS**

### **2.1. Field insect sampling and rearing**

A total of 12 CEW populations were collected from Louisiana, Florida, and Georgia in 2017 (Table 1). Among these, nine populations were collected from Louisiana, two populations were from Florida and one population was from Georgia. The nine Louisiana populations were sampled from Bt or non-Bt corn fields in three locations in Louisiana: Franklin Parish in northeast Louisiana, Rapides Parish in central Louisiana, and Tensas Parish in northeast Louisiana. These nine populations were LAF-NBt1, LAF-NBt2, LAF-NB3, LAF-Bt1, NAF-Bt2, LAF-Bt3, LAT-NBt, LAR-NBt and LAR-Bt. Population LAF- NBt1 was collected from refuge ears of non-Bt plants in a field of 90% Bt corn mixed with 10% non-Bt corn (90:10% RIB) near Winnsboro in Franklin Parish, LA. The Bt corn plants contained the Genuity<sup>®</sup>SmartStax<sup>®</sup> (SMT) trait, which expressed Cry1A.105, Cry2Ab2, and Cry1F for controlling above-ground lepidopteran pests including CEW and Cry3Bb1, Cry34/35Ab1 for managing under-ground rootworms (DiFonzo and Porter 2018). LAF-NBt population was sampled from the non-Bt refuge ears in a 80:20% RIB planting of SMT and non-Bt corn, while LAF-NBt3 was sampled from a field planted with pure non-Bt corn at the same location as LAF-NBt1. Likewise, insects from Bt plants of 90:10% and 80:20% SMT RIB plantings near Winnsboro constituted the populations LAF-Bt1, and LAF-Bt2, respectively. LAF-Bt3 was collected from a YieldGard corn field near Winnsboro. YieldGard corn expressed a single Bt protein, Cry1Ab (DiFonzo and Porter 2018). LAT-NBt was collected from a pure non-Bt corn field near St. Joseph in Tensas Parish. LAR-NBt was collected from a field planted with pure non-Bt corn near Alexandria in Rapides Parish, LA, while LAR-Bt was sampled from a pure SMT Bt corn field in the same area as LAR-NBt population. The two Florida populations were FL-A and FL-B. FL-A was collected

from pure non-Bt plants near Jay in Santa Rosa County in west Florida, while FL-B was collected from Bt plants containing the Genuity VT Double Pro trait at the similar lactation as FL-A. The Georgia population was named GA, which was from SMT Bt plants near Tifton in Tifton County, Georgia. In the sampling, 45-93 individuals of 2nd to 5th instars of CEW were collected from corn ears in each sampling. Field-collected larvae were individually reared in 30-ml plastic cups (Fill-Rite, Newark, NJ) containing a meridic diet (Ward's Stonefly Heliothis diet, Rochester, NY). The larval-rearing cups were held in 30-well trays (Bio-Serv, Frenchtown, NJ) and the trays were placed in a walk-in insect rearing room maintained at ~26°C with a 14 L:10 D photoperiod and ~50% r. h. Larvae survived well and few larvae were dead during the laboratory rearing. Pupae of each population collected from the insect rearing cups were placed into each 20-L mesh cage (Seville Classics, INC., Torrance, CA) containing ~300 g vermiculite (Sun Gro, Pine Bluff, AR) and 10% honey water solution. Insect development within population was synchronized by justifying temperatures during the pupal stage. The top of the cage containing pupae was covered with muslin cloth for adult egg-laying. The cages were then placed in incubators at 26°C, >70% RH and a 14:10 h (L:D) photoperiod for adult emergence, mating, and oviposition (Yang et al. 2014). In 2-3 days, females started laying eggs and the eggs laid on the muslin cloth were collected once a day. Muslin cloth containing eggs were kept in plastic bags for further use.

## **2.2. Sources of Cry1A.105 and Cry2Ab2 Bt proteins**

Cry1A.105 protein used in the study was solvated in a buffer solution, while lyophilized Cry2Ab2 corn leaf powder was the source of the Cry2Ab2 protein. Both Cry2Ab2 solution and Cry2Ab2 leaf powder, along with the related buffer solution and isoline non-Bt corn leaf powder were provided by Monsanto (St. Louis, MO).

Table 2.1. Sources of corn earworm populations sampled in three southwestern states of the U.S.

Population notation	Location, parish/county	Planting pattern	Ear source of larvae collected	No. larvae collected	Larval stages collected	Generations in the lab assayed
SS-BZ	A known laboratory susceptible colony to Bt proteins which was obtained from Benzon Research Inc., Carlisle, PA.					
LAF-NBt1	Franklin, LA	90:10% RIB	Non-Bt	78	3rd – 5th	F1
LAF-NBt2	Franklin, LA	80:20% RIB	Non-Bt	90	3rd – 5th	F1
LAF-NBt3	Franklin, LA	Pure non-Bt	Non-Bt	93	3rd – 5th	F1
LAF-Bt1	Franklin, LA	90:10% RIB	SMT Bt	65	3rd – 5th	F2
LAF-Bt2	Franklin, LA	80:20% RIB	SMT Bt	60	3rd – 5th	F2
LAF-Bt3	Franklin, LA	Pure Cry1Ab	YG Bt	55	3rd – 5th	F3
LAT-NBt	Tensas, LA	Pure non-Bt	Non-Bt	88	3rd – 5th	F1
LAR-NBt	Rapides, LA	Pure non-Bt	Non-Bt	66	3rd	F1
LAR-Bt	Rapides, LA	Pure Bt	SMT Bt	43	2nd	F2
FL-A	Santa Rosa, FL	Non-Bt	Non-Bt	62	3rd – 5th	F2
FL-B	Santa Rosa, FL	Bt	Pyramided Bt	45	3rd – 5th	F6
GA	Tifton, GA	Bt	SMT Bt	52		F2 & F3

### **2.3. Bioassay**

Susceptibility of the CEW populations listed in Table 1 was determined using a diet over-lay bioassay method as described in Marçon et al. 1999. In each bioassay, seven concentrations of a Bt protein were used: 0.01, 0.0316, 0.1, 0.316, 1, 3.16, and 10  $\mu\text{g}/\text{cm}^2$ . Bt protein solutions were prepared with 0.1% Triton X-100 nonionic detergent to obtain uniform spreading over the diet surface. Bioassays were performed in 128-cell trays (CD International, Pitman, NJ). In the bioassay, approximately 0.8 ml of a liquid diet (Southland Products, Lake Village, AR) were placed into each cell of the 128-cell trays using syringes (Becton, Dickinson and Company, Franklin Lakes, NJ). An amount of 50  $\mu\text{L}$  (for assaying Cry1A.105) or 200  $\mu\text{L}$  (for assaying Cry2Ab2) of appropriate concentration of Bt protein solution was applied on the diet surface in each cell (Marçon et al., 1999). A negative control (containing buffer for assaying Cry1A.105 or non-Bt leaf powder for assaying Cry2Ab2 only) and a blank control treated 0.1% Triton solution only were also included in each bioassay. After the diet treated with the Bt solution dried, one neonate (< 24 h) of a population was released on the diet surface in each cell. After larval inoculation, cells were covered with vented lids (C-D International, Pitman, NJ). The bioassay trays were placed in an environmental chamber maintained at 26  $^{\circ}\text{C}$ , ~50% RH, and a 16:8 (L:D) h photoperiod. Larval mortality was recorded on the 7<sup>th</sup> day after neonate release. In each bioassay, there were four replications with 16-32 larvae in each replicate.

### **2.4. Data analysis**

Original larval mortality at each Bt concentration was corrected based on the mortality observed in the negative control treatment (Abbott, 1925). The corrected dose/mortality data were then subjected to probit analysis to calculate the median lethal concentrations ( $\text{LC}_{50\text{s}}$ ) and the corresponding 95% confidence intervals (CI) (SAS Institute, 2010). For several populations,

larval mortalities were low, <50% across all seven tested Bt concentrations. The LC<sub>50</sub> value of these populations was considered to be >10 µg/cm<sup>2</sup> because its mortality at the Bt concentration of 10 µg/cm<sup>2</sup>, the highest concentration assayed in the study, was less than 50%. Resistance ratio of a field-collected CEW population to a Bt protein was calculated based on the LC<sub>50</sub> value of the population divided by the LC<sub>50</sub> of the known Bt susceptible population, SS-BZ. In addition, because the probit analysis couldn't be used to analyze the mortality data of some populations that had a low mortality, the corrected mortality data at the two highest Bt concentrations, 3.16 and 10 µg/cm<sup>2</sup>, were transformed using the arcsin (x)<sup>0.5</sup> to normalize the data. The transformed data were also analyzed using a one-way analysis of variance at each of the two Bt concentration with insect population as the main factor. Treatment means were separated using Tukey's HSD tests at  $\alpha = 0.05$  level (SAS Institute, 2010).



### 3. RESULTS

#### 3.1. Susceptibility of different populations of corn earworm to Cry1A.105 protein

The laboratory population, SS-BZ, was susceptible to Cry1A.105 protein in the diet over-lay bioassay. At the Cry1A.105 concentrations of 3.16 and 10  $\mu\text{g}/\text{cm}^2$ , > 96% larvae of SS-BZ were killed after 7 days of neonate release (Figs. 1 & 2) The calculated  $\text{LC}_{50}$  value of Cry1A.105 for SS-BZ was 0.011  $\mu\text{g}/\text{cm}^2$  with a 95% CI of 0.009 to 0.013 (Table 2). In contrast, susceptibility to the Cry1A.105 protein varied greatly among the 12 field collected CEW populations. Five out of the six populations collected from Franklin Parish in northeast Louisiana (LAF-NBt1, LAF-NBt3, LAF-Bt1, LAF-Bt2, and LA-FBt3) appeared to be highly resistant to the Cry1A.105 protein. Larval mortalities at the two highest Cry1A.105 concentrations (3.16 and 10  $\mu\text{g}/\text{cm}^2$ ) were less than 50% for all the five populations, ranged from 8.0 to 38.4% at 3.16  $\mu\text{g}/\text{cm}^2$  and from 17.6 to 40% at 10  $\mu\text{g}/\text{cm}^2$ . The observed mortalities of the five population were all significantly less ( $P \leq 0.05$ ) than the mortality of SS-BZ for both Cry1A.105 concentrations. The difference in the larval mortality at each of the two Bt concentrations was not significant ( $P > 0.05$ ) among the five field-collected populations.

As mentioned above, because the larval mortality of the five populations was < 50%, even at the highest Cry1A.105 concentration tested (10  $\mu\text{g}/\text{cm}^2$ ), their  $\text{LC}_{50}$  values were estimated to be >10  $\mu\text{g}/\text{cm}^2$  for all the five populations, which corresponded to a resistance ratio of > 909-fold, relative to the  $\text{LC}_{50}$  of SS-BZ (Table 3). LAF-NBt2, which was collected from Franklin Parish, LA, also demonstrated some levels of resistance to the Cry1A.105 protein. Larval mortality of LAF-NBt2 after 7 days of neonate release was 53.4% at 3.16  $\mu\text{g}/\text{cm}^2$  and 72.2% at 10  $\mu\text{g}/\text{cm}^2$ . The difference in the mortality, compared to SS-BZ, was significant ( $P \leq 0.05$ ) at 3.16  $\mu\text{g}/\text{cm}^2$ , while it was not significant ( $P > 0.05$ ) at 10  $\mu\text{g}/\text{cm}^2$ . The calculated  $\text{LC}_{50}$  value of Cry1A.105 for

LAF-NBt2 was 1.41  $\mu\text{g}/\text{cm}^2$  with a 95% CI of 0.60 to 4.05. The 128-fold difference in the  $\text{LC}_{50}$ s between SS-BZ and LAF-NBt2 was significant based on their non-overlapped 95% CIs.

The CEW population (LAT-NBt) collected from non-Bt corn plants in Tensas Parish in northeast Louisiana also showed a significant resistance level to the Cry1A.105 protein in the diet over-lay bioassay. Larval mortalities of LAT-NBt at 3.16 and 10  $\mu\text{g}/\text{cm}^2$  were 48.4 and 60.1%, respectively (Fig. 1 & 2), which were significantly less ( $P \leq 0.05$ ) than the mortality of SS-BZ for both Cry1A.105 concentrations and, in general, not significant ( $P > 0.05$ ) compared to the mortalities of the six populations collected from Franklin Parish mentioned above. The calculated  $\text{LC}_{50}$  value of Cry1A.105 for LAT-NBt was 3.64  $\mu\text{g}/\text{cm}^2$  with a 95% CI of 1.92 to 9.52, which was 331-fold of the  $\text{LC}_{50}$  of SS-BZ. The difference in the  $\text{LC}_{50}$ s between LAT-NBt and SS-BZ was significant based on the non-overlapped 96% CI of the  $\text{LC}_{50}$  values. In contrast, the two CEW populations collected from Rapides Parish in central Louisiana (LAR-NBt and LAR-Bt) were relatively more susceptible to the Cry1A.105 protein. Larval mortality of LAR-NBt was 96.5% at 3.16 and 10  $\mu\text{g}/\text{cm}^2$ , which was similar ( $P > 0.05$ ) to the mortality of SS-BZ and, in most cases, was significantly greater ( $P \leq 0.05$ ) than the mortalities of the Franklin and Tensas populations described above. The calculated  $\text{LC}_{50}$  of Cry1A.105 for LAR-NBt was 0.17  $\mu\text{g}/\text{cm}^2$  with a 95% CI of 0.11 to 0.26, which corresponded a resistance ratio of 15-fold, relative to SS-BZ. The 15-fold difference was significant based on their non-overlapped 95% CI of the  $\text{LC}_{50}$  values. Compared to LAR-NBt, the population collected from SMT Bt plants in Rapides Parish (LAR-Bt) was relatively more tolerant to the Cry1A.105 protein. Larval mortalities of LAR-Bt at 3.16 and 10  $\mu\text{g}/\text{cm}^2$  were 48.4 and 60.1%, respectively. The difference in the larval mortalities between the two Rapides populations was not significant ( $P > 0.05$ ) at 3.16  $\mu\text{g}/\text{cm}^2$ , but significant ( $P \leq 0.05$ ) at 10  $\mu\text{g}/\text{cm}^2$ . The calculated  $\text{LC}_{50}$  value of Cry1A.105 for LAR-Bt was

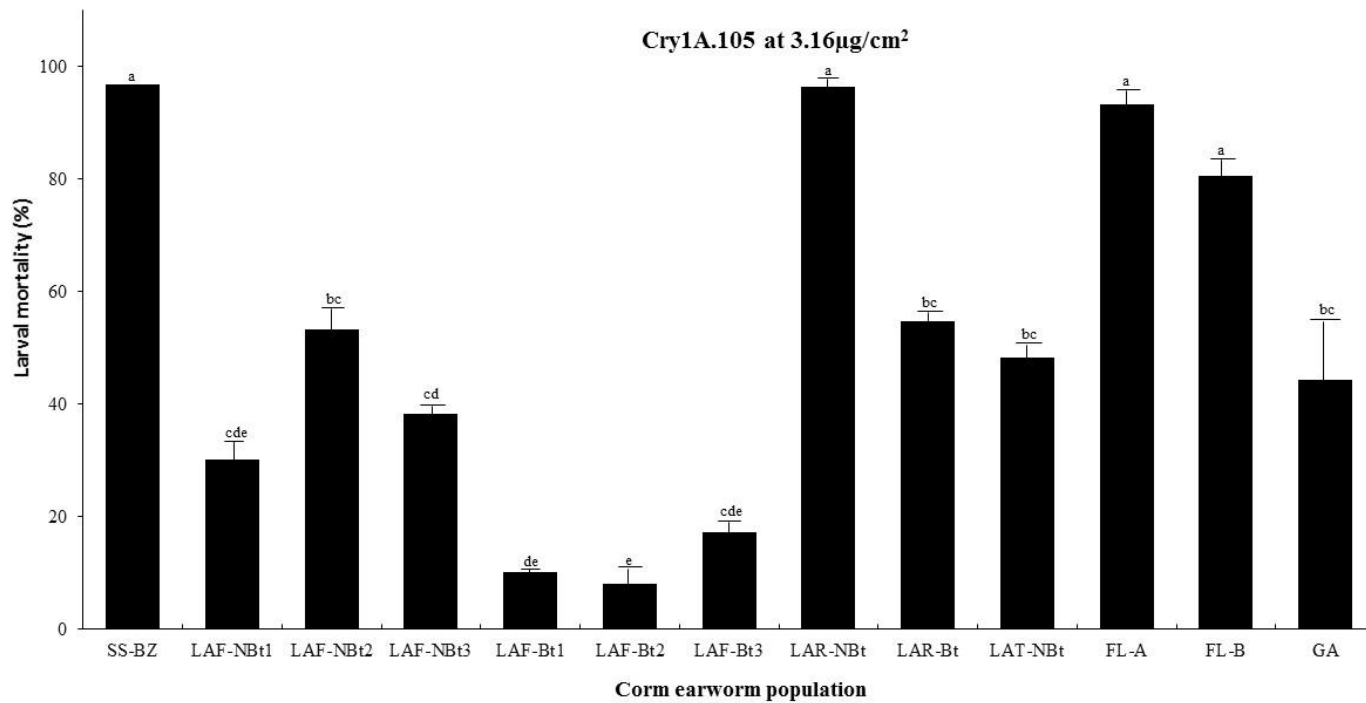


Figure 2.1. Corrected larval mortality (% mean  $\pm$  sem) of corn earworm populations collected from multiple locations in Louisiana, Florida and Georgia after 7 days on diet treated with Cry1A.105 protein at the concentration of 3.16  $\mu\text{g}/\text{cm}^2$ . Mean values in a figure followed by a same letter are not significantly different (Tukey's HSD test,  $\alpha = 0.05$ ).

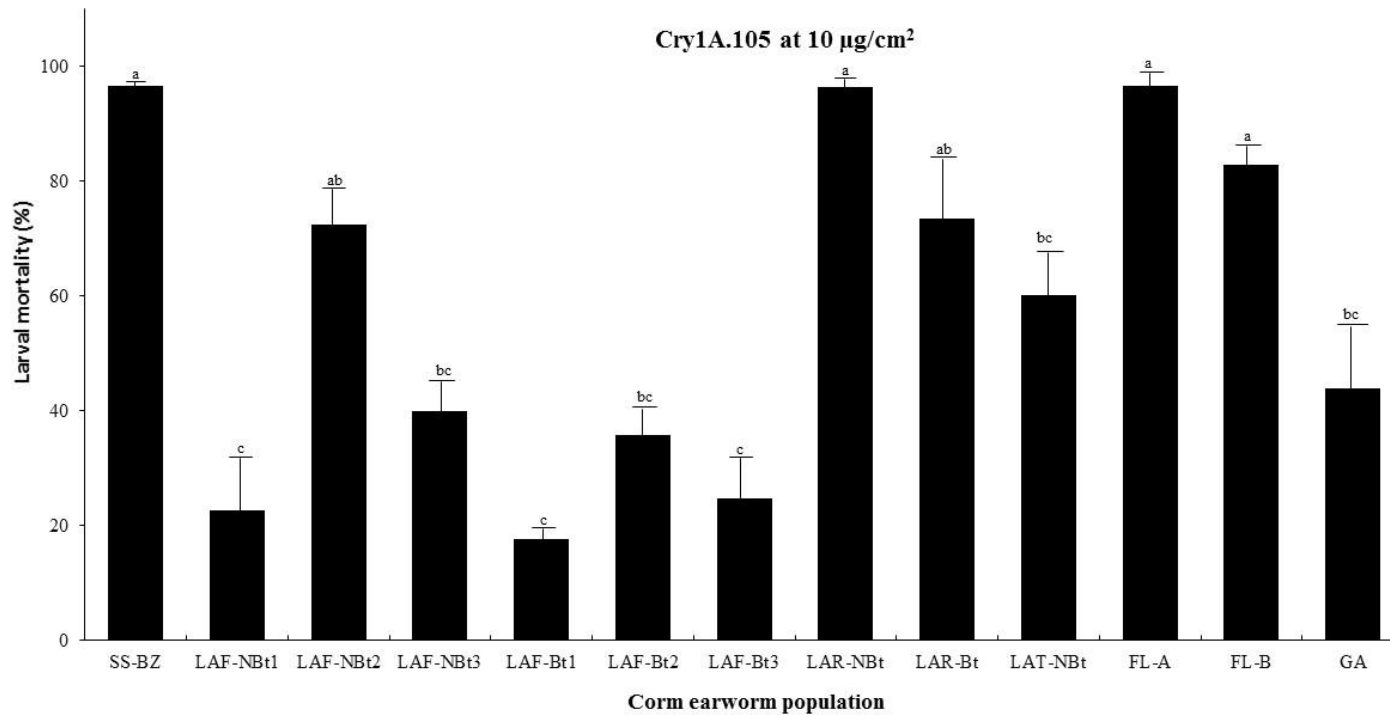


Figure 2.2. Corrected larval mortality (% , mean ± sem) of corn earworm populations collected from multiple locations in Louisiana, Florida and Georgia after 7 days on diet treated with Cry1A.105 protein at the concentration of 10 µg/cm<sup>2</sup>. Mean values in a figure followed by a same letter are not significantly different (Tukey's HSD test, α = 0.05).

Table 2.2. Susceptibility of corn earworm collected from multiple locations in Louisiana, Florida and Georgia to Cry1A.105 protein

Population	No. neonates assayed	Slope $\pm$ SE	LC <sub>50</sub> (95% CI, or larval mortality at 10 $\mu$ g/cm <sup>2</sup> )	$\chi^2$	P-value	Resistance ratio*
SS-BZ	1129	2.1 $\pm$ 0.2	0.011(0.009, 0.013)	9.5	0.4856	---
LAF-NBt1	631	n/a	>10 (22.6%)	n/a	n/a	> 909
LAF-NBt2	1129	0.65 $\pm$ 0.16	1.41 (0.60, 4.05)	78.9	0.0001	128
LAF-NBt3	544	n/a	>10 (40.0%)	n/a	n/a	> 909
LAF-Bt1	623	n/a	>10 (17.8%)	n/a	n/a	> 909
LAF-Bt2	585	n/a	>10 (35.9%)	n/a	n/a	> 909
LAF-Bt3	565	n/a	>10 (24.7%)	n/a	n/a	> 909
LAT-NBt	1112	0.56 $\pm$ 0.08	3.64 (1.92, 9.52)	40.9	0.0084	331
LAR-NBt	544	1.3 $\pm$ 0.15	0.17 (0.11, 0.26)	54.9	0.0001	15
LAR-Bt	576	0.35 $\pm$ 0.08	0.39 (0.12, 1.38)	45.0	0.0119	35
FL-A	573	1.18 $\pm$ 0.21	0.093 (0.039, 0.169)	68.9	0.0001	8
FL-B	742	0.57 $\pm$ 0.06	0.19 (0.12, 0.96)	21.5	0.7146	17
GA	504	n/a	>10 (43.9%)	n/a	n/a	>909

\* Resistance ratio of a field-collected insect population to the Bt protein was calculated based on the LC<sub>50</sub> value of the population divided by the LC<sub>50</sub> of the known Bt susceptible population, SS-BZ. LC value of a field-collected population was considered to be >10  $\mu$ g/cm<sup>2</sup> if its mortality at the Bt concentration of 10  $\mu$ g/cm<sup>2</sup> was less than 50% in the bioassay.

0.39  $\mu\text{g}/\text{cm}^2$  with a 95% CI of 0.12 to 1.38, which was not significantly different ( $P > 0.05$ ) than the  $\text{LC}_{50}$  of LAR-NBt based on their over-lapped 95% CIs. The 35-fold resistance ratio of LAR-Bt, relative to SS-BZ, was significant based on their non-overlapped 95% CIs of the  $\text{LC}_{50}$  values.

Compared the populations from Franklin and Tensas, LA, the two CEW populations collected from Florida (FL-A and FL-B) was generally more susceptible to the Cry1A.105 protein in the diet over-lay bioassay, while they performed similarly to the populations from Rapides Parish. Larval mortalities of FL-A and FL-B was 93.4 and 80.5 at 3.16  $\mu\text{g}/\text{cm}^2$ , and 96.7 and 82.8 at 10  $\mu\text{g}/\text{cm}^2$ , respectively, which was similar ( $P > 0.05$ ) to the mortalities of SS-BZ for both Bt concentrations. The mortalities of the two Florida populations were, in generally, significantly greater ( $P \leq 0.05$ ) than those of the seven populations collected from Franklin and Tensas, LA. The calculated  $\text{LC}_{50}$ s of Cry1A.105 for FL-A was 0.093  $\mu\text{g}/\text{cm}^2$  with a 95% CI of 0.039 to 0.169, which corresponded to a resistance ratio of 8-fold, relative to the  $\text{LC}_{50}$  of SS-BZ. The 8-fold difference in the  $\text{LC}_{50}$ s between FL-A and SS-BZ was significant based on their non-overlapped 95% CIs. The corresponded  $\text{LC}_{50}$  value of FL-B was 0.19  $\mu\text{g}/\text{cm}^2$  with a 95% CI of 0.12 to 0.96. The 17-fold difference in the  $\text{LC}_{50}$  value, relative to the  $\text{LC}_{50}$  of SS-BZ, was significant based on their non-overlapped 95% CIs. However, the difference in the  $\text{LC}_{50}$ s between the two Florida populations was not significant based on their overlapped 95% CIs. The CEW population collected from SMT Bt plants in Georgia (GA) was also highly resistant to the Cry1A.105 protein. Larval mortality of GA was about 44% at 3.16 and 10  $\mu\text{g}/\text{cm}^2$ , which was significantly less ( $P \leq 0.05$ ) than the mortalities of SS-BZ and, in most cases, was similar to the mortalities of the seven populations collected from northeast Louisiana. Similarly, as mentioned above, because the mortality at the highest Cry1A.105 concentration, 10  $\mu\text{g}/\text{cm}^2$ , was only 43.9

(< 50%), the LC<sub>50</sub> for GA was considered to be >10 µg/cm<sup>2</sup>, which resulted in a resistance ratio of > 909-fold, relative to the LC<sub>50</sub> of SS-BZ.

### **3.2. Susceptibility of different populations of corn earworm to Cry2Ab2 protein**

The laboratory population, SS-BZ, was also susceptible to the Cry2Ab2 protein in the diet overlay bioassay. Larval mortality of SS-BZ after 7 days of neonate release was 88.7% at the Cry2Ab2 concentration of 3.16 µg/cm<sup>2</sup> and 99.1% at 10 µg/cm<sup>2</sup> (Figs. 3 & 4) The calculated LC<sub>50</sub> value of Cry2Ab2 for SS-BZ was 0.40 µg/cm<sup>2</sup> with a 95% CI of 0.29 to 0.54 (Table 3). Similarly, as observed in the bioassays with the Cry1A.105 protein, the susceptibility to the Cry2Ab2 protein also differed considerably among the 12 field collected populations. The five populations collected from Franklin Parish in LA (LAF-NBt1, LAF-NBt3, LAF-Bt1, LAF-Bt2, and LAF-Bt3) that exhibited highly resistant to the Cry1A.105 protein were also highly resistant to the Cry2Ab2 protein. Larval mortalities were similar ( $P > 0.05$ ) among the five populations, ranging from 0-36.2% at the Cry2Ab concentration of 3.16 µg/cm<sup>2</sup> and 28.5-64.5% at 10 µg/cm<sup>2</sup>, which were significantly less ( $P \leq 0.05$ ) than the mortalities of SS-BZ for both Bt concentrations (Fig. 3 & 4). Because the larval mortalities of LAF-NBt1, LAF-NBt3, LAF-Bt1, and LAF-Bt2 were less than 50% even at the highest Cry2Ab2 concentration assayed, 10 µg/cm<sup>2</sup>, their LC<sub>50</sub> values were considered to be >10 µg/cm<sup>2</sup> for the four populations, which corresponded to a resistance ratio of > 28-fold, relative to the LC<sub>50</sub> of SS-BZ (Table 3). The remaining population (LAF-Bt3) that was collected from Cry1Ab Bt plants had a LC<sub>50</sub> value of 7.01 µg/cm<sup>2</sup>, representing an 18-fold resistance ratio, relative to the LC<sub>50</sub> of SS-BZ. As observed in the bioassay with Cry1A.105, the population, LAF-NBt2 that was collected the refuge plants of an 80:20 RIB planting in Franklin Parish, LA, also showed some levels of resistance to the Cry2Ab2 protein. Larval mortality of LAF-NBt2 was 31.3% at 3.16 µg/cm<sup>2</sup> and 90.6% at 10

$\mu\text{g}/\text{cm}^2$ . The difference in the mortality, compared to SS-BZ, was significant ( $P \leq 0.05$ ) at  $3.16 \mu\text{g}/\text{cm}^2$ , while it was not significant ( $P > 0.05$ ) at  $10 \mu\text{g}/\text{cm}^2$ . The calculated  $\text{LC}_{50}$  value of Cry2Ab2 for LAF-NBt2 was  $3.61 \mu\text{g}/\text{cm}^2$  with a 95% CI of 2.23 to 6.84. The 9-fold difference in the  $\text{LC}_{50}$ s between SS-BZ and LAF-NB2 was significant based on their non-overlapped 95% CIs.

The population, LAT-NBt that was collected from non-Bt corn plants in Tensas Parish, LA was also highly resistant to the Cry2Ab2 protein in diet over-lay bioassay. Larval mortality of LAT-NBt was only 3.3% at  $3.16 \mu\text{g}/\text{cm}^2$  and 44.7%  $10 \mu\text{g}/\text{cm}^2$  (Fig. 3 & 4), which was significantly less ( $P \leq 0.05$ ) than the mortality of SS-BZ for both Bt concentrations. The larval mortalities of LAT-NBt at the two Bt concentrations were similar ( $P > 0.05$ ) to those of the five most resistant populations collected from Franklin Parish. Because the mortality of LAT-NBt was  $< 50\%$  at the highest Cry2Ab2 concentration assayed, its  $\text{LC}_{50}$  value of Cry2Ab2 was considered to be  $> 10 \mu\text{g}/\text{cm}^2$ , which represented a resistance ratio of 25-fold, relative to the  $\text{LC}_{50}$  of SS-BZ. The two CEW populations collected from Rapides Parish, LA (LAR-NBt and LAR-Bt) showed a low level of resistance to Cry2Ab2 protein. Larval mortality of LAR-NBt was 43.6% at  $3.16 \mu\text{g}/\text{cm}^2$  and 82.5% at  $10 \mu\text{g}/\text{cm}^2$ . Compared to SS-BZ, the difference in the larval mortality was significant ( $P \leq 0.05$ ) for the concentration of  $3.16 \mu\text{g}/\text{cm}^2$ , but not significant ( $P > 0.05$ ) at  $10 \mu\text{g}/\text{cm}^2$  similar. The calculated  $\text{LC}_{50}$  of Cry2Ab2 for LAR-NBt was  $2.54 \mu\text{g}/\text{cm}^2$  with a 95% CI of 1.26 to 6.84, which corresponded a resistance ratio of 6-fold, relative to SS-BZ. The difference in the  $\text{LC}_{50}$ s between LAR-NBt and SS-BZ was significant based on their non-overlapped 95% CIs. Similarly, the population collected from SMT Bt plants in Rapides Parish (LAR-Bt) also showed some levels of resistance to the Cry2Ab2 protein in the bioassay. Larval mortalities of LAR-Bt were 54.4% at  $3.16 \mu\text{g}/\text{cm}^2$  and 59.9% at  $10 \mu\text{g}/\text{cm}^2$ . The



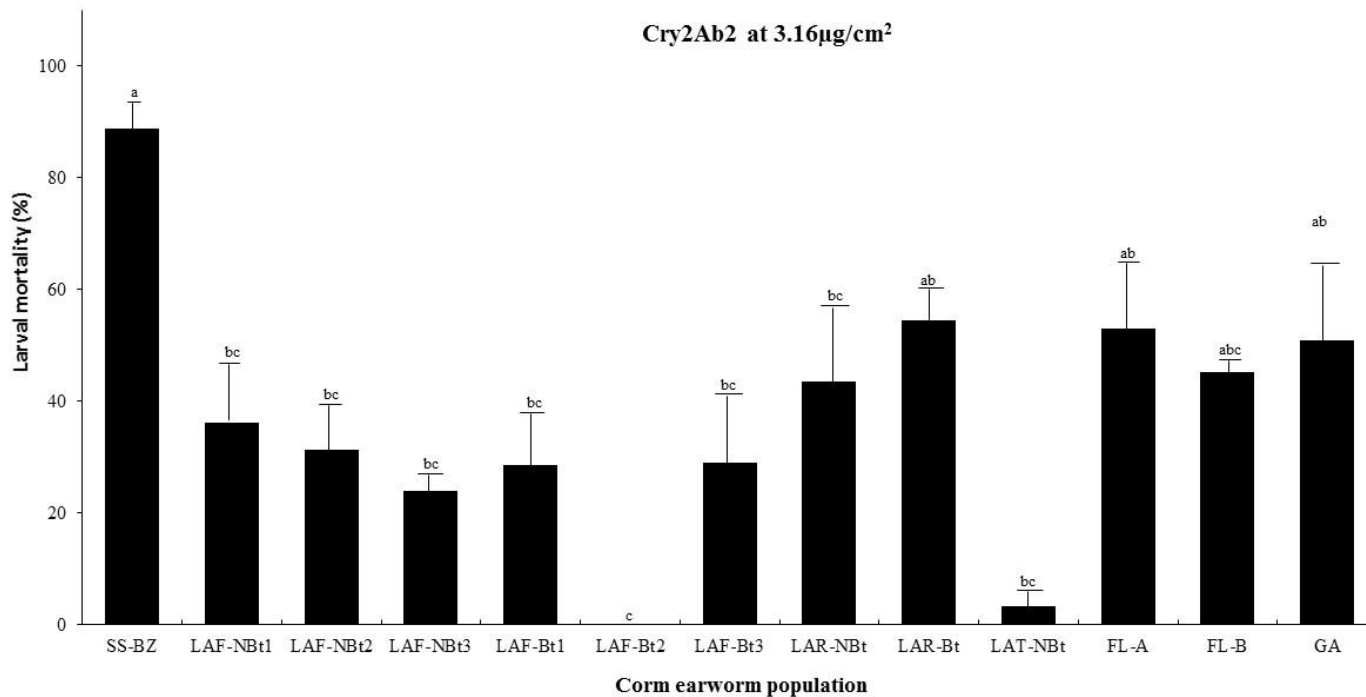


Figure 2.3. Corrected larval mortality (% mean  $\pm$  sem) of corn earworm populations collected from multiple locations in Louisiana, Florida and Georgia after 7 days on diet treated with Cry2Ab2 protein at the concentration of 3.16  $\mu\text{g}/\text{cm}^2$ . Mean values in a figure followed by a same letter are not significantly different (Tukey's HSD test,  $\alpha = 0.05$ ).

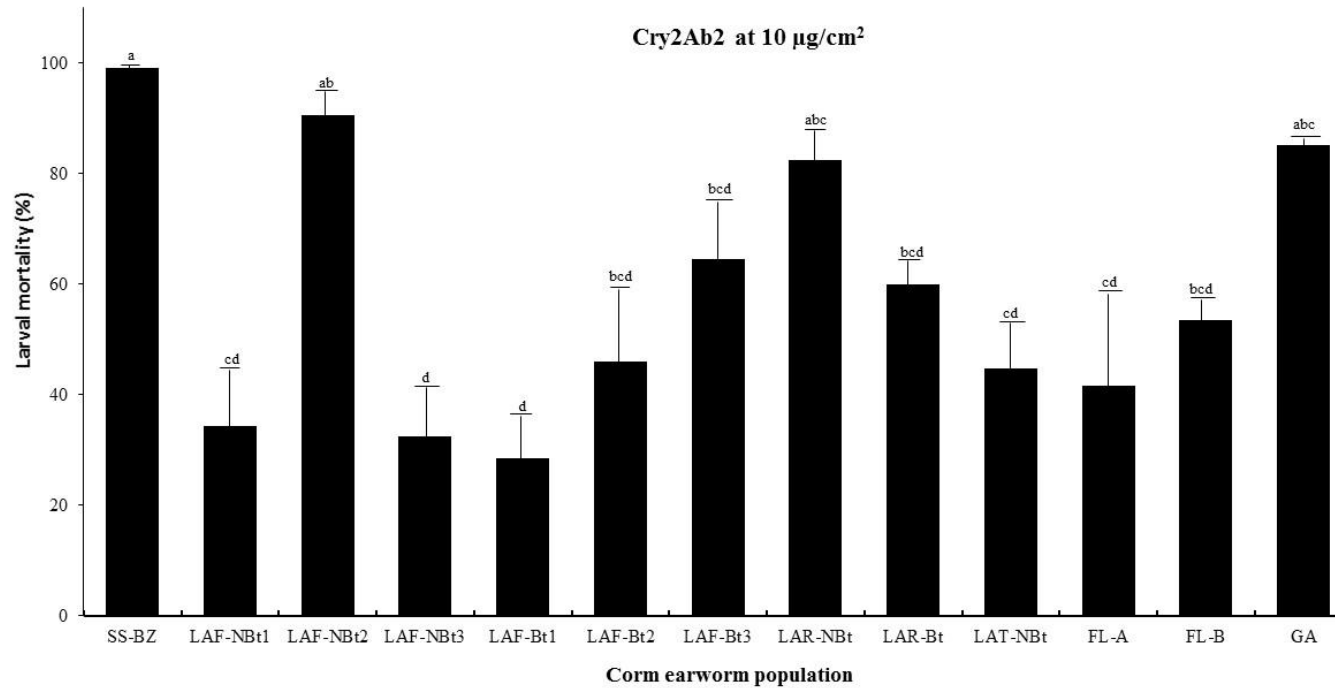


Figure 2.4. Corrected larval mortality (% , mean ± sem) of corn earworm populations collected from multiple locations in Louisiana, Florida and Georgia after 7 days on diet treated with Cry2Ab2 protein at the concentration of 10 µg/cm<sup>2</sup>. Mean values in a figure followed by a same letter are not significantly different (Tukey’s HSD test, α = 0.05).

Table 2.3. Susceptibility of corn earworm collected from multiple locations in Louisiana, Florida and Georgia to Cry2Ab2 protein

Population	No. neonates assayed	Slope $\pm$ SE	LC <sub>50</sub> (95% CI, or larval mortality at 10 $\mu$ g/cm <sup>2</sup> )	$\chi^2$	P-value	Resistance ratio*
SS-BZ	1101	1.56 $\pm$ 0.15	0.40 (0.29, 0.54)	56.4	0.0001	---
LAF-NBt1	906	n/a	>10 (34.3%)	n/a	n/a	> 25
LAF-NBt2	621	1.91 $\pm$ 0.39	3.61 (2.23, 6.84)	50.3	0.0001	9
LAF-NBt3	1151	n/a	>10 (32.5%)	n/a	n/a	> 25
LAF-Bt1	631	n/a	>10 (28.5%)	n/a	n/a	> 25
LAF-Bt2	298	n/a	>10 (46.1%)	n/a	n/a	> 25
LAF-Bt3	574	0.64 $\pm$ 0.29	7.01 (2.00, )	55.4	0.0002	18
LAT-NBt	971	n/a	>10 (44.7%)	n/a		> 25
LAR-NBt	557	1.15 $\pm$ 0.29	2.54 (1.26, 6.84)	43.9	0.0001	6
LAR-Bt	576	0.78 $\pm$ 0.13	3.68 (2.17, 8.36)	16.3	0.4317	9
FL-A	288	0.50 $\pm$ 0.24	3.78 (0, )	43.9	0.0001	9
FL-B	1046	0.74 $\pm$ 0.09	6.04 (3.40, 13.58)	27.1	0.0773	15
GA	571	0.96 $\pm$ 0.28	1.88 (0.72, 5.16)	45.6	0.0001	5

\* Resistance ratio of a field-collected insect population to the Bt protein was calculated based on the LC<sub>50</sub> value of the population divided by the LC<sub>50</sub> of the known Bt susceptible population, SS-BZ. LC value of a field-collected population was considered to be >10  $\mu$ g/cm<sup>2</sup> if its mortality at the Bt concentration of 10  $\mu$ g/cm<sup>2</sup> was less than 50% in the bioassay.

difference in the larval mortalities between LAR-Bt and SS-BZ was significant ( $P \leq 0.05$ ) at 10  $\mu\text{g}/\text{cm}^2$ , but not significant ( $P > 0.05$ ) at 3.16  $\mu\text{g}/\text{cm}^2$ . The difference in larval mortality between the two Rapides, LA was not significant ( $P > 0.05$ ) at each of the two concentrations. The calculated  $\text{LC}_{50}$  value of Cry2Ab2 for LAR-Bt was 3.68  $\mu\text{g}/\text{cm}^2$  with a 95% CI of 2.17 to 8.36, which was not significantly different than the  $\text{LC}_{50}$  of LAR-NBt based on their over-lapped 95% CIs, while the 9-fold resistance ratio of LAR-Bt, relative to SS-BZ, was significant based on their non-overlapped 95% CIs of the  $\text{LC}_{50}$  values.

The two CEW populations collected from Florida (FL-A and FL-B) also exhibited some levels of resistance to the Cry2Ab2 protein in the diet over-lay bioassay. Larval mortalities of FL-A and FL-B was 51.3 and 45.3% at 3.16  $\mu\text{g}/\text{cm}^2$ , and 41.7 and 53.6% at 10  $\mu\text{g}/\text{cm}^2$ , respectively, which were similar ( $P > 0.05$ ) to the mortality of SS-BZ at the concentration of 3.16  $\mu\text{g}/\text{cm}^2$ , but significant ( $P \leq 0.05$ ) less than that of SS-BZ at 10  $\mu\text{g}/\text{cm}^2$  for both populations. The mortalities of the two Florida populations were also not significantly different ( $P > 0.05$ ) compared to the mortalities of the seven populations collected from Franklin and Tensas, LA. The calculated  $\text{LC}_{50}$ s of Cry1A.105 for FL-A was 3.78  $\mu\text{g}/\text{cm}^2$ , which corresponded to a resistance ratio of 9-fold, relative to the  $\text{LC}_{50}$  of SS-BZ. The corresponded  $\text{LC}_{50}$  value of FL-B was 6.04  $\mu\text{g}/\text{cm}^2$  with a 95% CI of 3.40 to 13.58. The 15-fold difference in the  $\text{LC}_{50}$  values, relative to the  $\text{LC}_{50}$  of SS-BZ, was significant based on their non-overlapped 95% CIs. The CEW population collected from SMT Bt plants in Georgia (GA) exhibited a low level of resistance to the Cry1A.105 protein in the bioassay. Larval mortalities of GA were about 50.8% at 3.16  $\mu\text{g}/\text{cm}^2$  and 85.2% at 10  $\mu\text{g}/\text{cm}^2$ , which were not significantly different ( $P > 0.05$ ) compared to the mortalities of SS-BZ for both Bt concentrations. The calculated  $\text{LC}_{50}$  of Cry2Ab2 for GA was 1.88  $\mu\text{g}/\text{cm}^2$  with a

95% CI of 0.72 to 5.16. The 5-fold difference between GA and SS-BZ was significant based on their non-overlapped 95% CIs of the LC<sub>50</sub> values.

### **3.3. DISCUSSION**

CEW is a polyphagous pest. It can complete its life cycle on many hosts, which makes its management more difficult. Different management practices have been adopted to control this pest, but using Bt crops is one of the most effective methods. Bt crops are environmental-friendly, safe to mammals, animals, birds, human, and natural enemies. Since 1996, Bt crops including Bt corn and Bt cotton have been intensively planted in the U.S. In Louisiana, Reduced control efficacy was observed in 2016 in some fields planted with Bt corn containing Genuity® VT Double Pro® or SMT. VT Double Pro® corn plants contain the Bt event MON 89034, which is a pyramided Bt trait expressing both Cry1A.105 and Cry2Ab2 proteins for controlling moth pests including CEW. Bt products producing these proteins were very effective against CEW before 2016 in Louisiana. In 2017, several field experimental plots planted with SMT in Franklin Parish were heavily infested with CEW. Laboratory bioassays of this study showed that CEW populations collected from these fields with the control problem were considerably less sensitive to the Bt proteins Cry1A.105 and Cry2Ab2 which were expressed in the Bt corn planted in these fields. More importantly, the insect population (LAW-NBt3) collected from the field planted with pure non-Bt corn was also highly resistant to both Bt proteins. The results of the bioassays from this study validated that the field control problem of SMT observed in the fields in Franklin Parish in northeast Louisiana was due to resistance development of CEW to the Bt proteins expressed in the plants. This is the first documentation of field resistance to Bt corn in any target insect species in the mid-south region of the U.S. Another target pest, the fall armyworm has

developed field resistance to Cry1F Bt corn in some states of the south-eastern region of the U.S. including Florida and North Carolina (Huang et al., 2014; Li et al., 2016).

In addition, the population (LAT-NBt) collected from the field with pure stand of non-Bt corn in Tensas Parish in northeast Louisiana, which is approximately 60 km away from the sampling fields in Franklin Parish, was also highly resistant to both the Cry1A.105 and Cry2Ab2. The results suggest that the field resistance of CEW to these two Bt proteins in the transgenic plants was likely common in the northeast area of the state. Compared to the populations from the northeast Louisiana, the two CEW populations (LAR-NBt and LAR-Bt) collected from Rapides Parish in the central region of the state were more sensitive to both Cry1A.105 and Cry2Ab2 and showed only a relatively low level of resistance to the two proteins. Transgenic Bt corn containing these Bt proteins has been effective and no field control problem has been reported from this region. The results indicate that resistance of CEW to the Bt corn containing Cry1A.105/Cry2Ab2 likely hasn't reached the level that causes field control problem the sampling area in Rapides Parish. However, data of the laboratory bioassay showed that the population (LAR-Bt) from Bt corn field was less sensitive to both Cry1A.105 and Cry2Ab2 than the population (LAR-NBt) from non-Bt plants, indicating that strong selection for resistance to the two proteins is likely on-going in this area. The two populations (FL-A and FL-B) collected from Florida was relatively more sensitive to the Cry1A.105 protein, but exhibited a similar susceptibility to the Cry2Ab2 protein as the populations from Rapides Parish, LA. Similarly, the population from Bt corn fields (FL-B) was less susceptible to both Bt proteins than the population (FL-A) sampled from non-Bt fields, suggesting strong on-going field selection of resistance in the area. In contrast, the population (GA) from Bt corn field in Georgia was highly resistant to Cry1A.105 but relatively susceptible to the Cry2Ab2 protein. The variable

susceptibility of CEW populations from different areas of the three southern states might indicate a mosaic pattern of the resistance in the region.

Transgenic corn containing pyramided Bt proteins of Cry1A.105 and Cry2Ab was first commercially planted in 2010 in the U.S. including the southern region. Many reasons might have contributed to the rapid development of resistance in CEW in the region. Firstly, Bt proteins expressed in Bt corn and Bt cotton are similar, and Cry2Ab2 is also a common Bt protein expressed in most Bt corn and Bt cotton varieties that have planted in the U. S. (US-EPA, 2012). Although Cry1A.105 is not expressed in Bt cotton plants, it is a chimeric protein consisting of Cry1Ab, Cry1Ac, and Cry1F ((Biosafety Clearing-House, 2009). Thus, Cry1A.105 is structurally similar to several other Cry1 proteins expressed in Bt corn and Bt cotton such as Cry1Ab, Cry1F, and Cry1Ac. In the southern region of the U.S., CEW is a cross-crop target pest of both Bt corn and Bt cotton (Yang et al., 2016). Each year, after corn is not suitable for CEW, it moves to cotton (and other crops) and continue for 2-3 more generations, mainly on cotton plants, in the region (US-EPA, 2010). This kind of ecosystem in the southern region, coupled with the cross-crop pest behavior and similar Bt proteins in Bt corn and Bt cotton, should have created an environment that causes CEW multiple exposures to Bt proteins expressed in the two crops each year. The northeast region of Louisiana where CEW has shown highly resistant to SMT plants is also the major area of the state where both Bt corn and Bt cotton have widely been planted since 1996 (cotton) and 1999 (corn). Secondly, because the similar structures in the Cry1 proteins expressed in the Bt plants, studies have shown that there are strong cross-resistances between Cry1A.105 and other Cry1 proteins (Niu et al., 2013; 2014; 2016; Huang et al., 2014; Yang et al., 2016; 2017a; 2017b). In addition, cross-resistance between Cry2Ab2 and Cry2Ae which is expressed in some Bt cotton varieties has also been documented (Yang et al.,

2017b). Thus, resistance to other Bt proteins can cause resistance to the Cry1A.105 or Cry2Ab2 in the SMT plants. A few early studies have shown that CEW populations collected from Cry1Ac cotton fields were less susceptible to Cry1Ac and Cry2Ab2 proteins in the laboratory bioassays (Ali et al., 2016; Ali and Luttrell, 2007; Luttrell and Ali, 2009). Such early selections with Bt cotton could also cause pre-selections before Cry1A.105/Cry2Ab2 corn was planted, and thus could accelerate resistance development in the field. Thirdly, theoretically, use of transgenic plants containing pyramided Bt genes that have different mode of actions could delay resistance development considerably (Zhao et al., 2003). However, the Bt proteins in the pyramided corn and cotton have been used sequentially, but not the same time. For example, before the pyramided Bt corn hybrids containing Cry1A.105 and Cry2Ab2 (MON 89034) were commercialized in 2010, Cry1Ab and Cry1F corn hybrids had been planted many years in the U.S. As mentioned above, due to the similarity in the gene structures, there is highly cross-resistance among Cry1A.105, Cry1Ab, and Cry1F. Thus, only the Cry2Ab protein in the pyramided MON 89034 corn was a ‘new’ protein and pre-selection for the Cry1A.105 protein had already existed for many years when MON 89034 was planted in 2010. Along with the pre-selection of Cry1Ac and Cry2Ab2 in Bt cotton, resistance allele frequencies to both Cry1A.105 and Cry2Ab2 should not be very rare in the CEW populations when Cry1A.105/Cry2Ab2 corn hybrids were first planted in 2010. Sequential use of Bt proteins in pyramided Bt crops might be a major reason that has contributed to the recent surging of resistance to pyramided Bt crops in other areas as well (Naik et al., 2018; Diverly et al., 2016; Tabashnik et al., 2017). In addition, CEW is a long-distance migratory insect. It can’t overwinter in the north region of the U.S. Each year, when weather becomes suitable and host crops are available in the north region, CEW migrates to the north and causes damage on the hosts such as corn. A recent study has shown that



CEW are highly resistant to transgenic sweet corn that contains pyramided Bt protein of Cry1A.105 and Cry2Ab2 in field populations in Maryland, U.S. (Diverly et al., 2016). Beside the possible local selections, resistant populations of CEW migrated from the south region could be the major contribution for the resistance to Cry1A.105/Cry2Ab2 sweet corn reported in Maryland.

The documentation of the field resistance to Cry1A.105/Cry2Ab2 proteins in the Bt plants should have important implication for development of effective resistance management strategies for the sustainable use of the Bt crop technologies. Because of the recent surging of resistance to Bt crops, a relatively new protein, Vip3, produced in the vegetative stages of *B. thuringiensis* has been incorporated into both transgenic Bt corn and Bt cotton (Yang et al., 2018; DiFonzo and Porter, 2018). Pyramided Bt corn and Bt cotton expressing Cry1, Cry2A, and Vip3A have recently become commercially available in the U.S. and several other countries. Several studies have shown that the Vip3A has a different mode of action than other Bt proteins in the plants; and Bt corn and Bt cotton plants expressing the Vip3A protein are still very effective against the Cry1/Cry2A resistance in several target species (Niu et al., 2013; 2014; 2016; Wanglia et al., 2012; Huang et al., 2014; Bernardi et al., 2015; Horikoshi et al., 2016; Yang et al., 2018). Thus, Bt plants containing the Vip3A protein should provide a means for managing the Cry1/Cry2A resistance in CEW. However, as mentioned above, these pyramided Bt crops containing Cry1, Cry2, and Vip3A would function as just the single-gene Bt plants and resistance development to these pyramided Bt crops could be quickly in the area where Cry1/Cry2-resistance has occurred in the insect. Additional management methods with different mortality factors are urgently needed to sustain the success of Bt crop technology as an effective pest management tool.

#### **4. CONCLUSIONS AND SUMMARY**

Results of the diet over-lay bioassays with the field-collected CEW populations validated that the field control problem of SMT Bt corn recently observed in northeast Louisiana was due to the development of resistance to the Cry1A.105 and Cry2Ab2 proteins in the plants.

Resistance/susceptibility levels to Cry1A.105 and Cry2Ab2 are still varied among CEW populations in the U.S. south-eastern region, which may indicate a mosaic distribution of the resistance in the region. The major reasons that caused the rapid development of resistance to the pyramided Cry1A.105/Cry2Ab2 corn in the region might include that 1) both Bt corn and Bt cotton are planted in the region; 2) CEW is a cross-crop pest of corn and cotton as well as a cross-crop target of both Bt corn and Bt cotton; 3) limited mode of action and similar Bt proteins are used in both Bt corn and Bt cotton; 4) strong cross-resistance exists among Bt proteins expressed in the Bt plants; and 5) different Bt proteins in the pyramided Bt crops have been introduced sequentially. The documentation of the field resistance to Cry1A.105/Cry2Ab2 Bt corn should have important implication for resistance management. Additional mode of actions against CEW is urgently needed to ensure the long-term success of the transgenic Bt crops in the southern region of the U.S.

##### **4.1. SUMMARY**

Corn earworm (CEW), *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), is a major pest of corn and cotton in the U. S. It is a polyphagous pest and can complete its life cycle on many hosts, which makes its management difficult. Bt crops (e.g. corn and cotton) containing pyramided genes were very effective to control this moth pest before 2016. However, in the past two years, significant CEW damage has been observed in some corn fields in northeast Louisiana. Especially in 2017, heavily ear damage and significantly larval survival of CEW

occurred on Bt corn plants containing pyramided Cry1A.105/Cry2Ab2 proteins in LSU AgCenter experimental station in Franklin Parish in northeast Louisiana. The main objective of this study was to determine if the field control problem of the Cry1A.105/Cry2Ab2 corn in northeast Louisiana against CEW was due to resistance development of the insect to Bt proteins in the plants. In addition, I also liked to generate some information about the possible distribution of the resistance in the southern region of the U.S.

To accomplish the proposed objectives, 12 CEW populations were collected from Bt and non-Bt corn plants in Louisiana, Florida, and Georgia. Among these, seven populations were collected from corn fields in northeast Louisiana which included three populations from Bt plants and four populations from non-Bt plants. Two populations were collected from central Louisiana, one from Bt corn plants and one from non-Bt corn plants. Two populations were sampled from western Florida, one from Bt plants and one from non-Bt plants. And one population was collected from Bt corn plants in Georgia. Field-collected CEW larvae were reared in a meridic diet in the laboratory. Susceptibility of the progeny neonates of the 12 field-collected populations, along with a known Bt-susceptible CEW strain, to the Cry1A.105 and Cry2Ab2 proteins were determined using a diet over-lay method at seven Bt concentrations: 0.01, 0.0316, 0.1, 0.316, 1, 1.316, and 10  $\mu\text{g}/\text{cm}^2$ . Larval mortality in the bioassays was checked after 7 days of neonate release. Resistance ratios of the field-collected populations were calculated by dividing the median lethal concentrations ( $\text{LC}_{50\text{S}}$ ) of the field-collected populations by the  $\text{LC}_{50}$  of the known Bt-susceptible strain.

Results of the laboratory bioassay showed that six out of the seven CEW populations collected from Bt and non-Bt corn fields in northeast Louisiana were highly resistant to both the Cry1A.105 and Cry2Ab2 proteins. Relative to the known Bt-susceptible strain, resistance ratio of the six populations was as high as >909-fold for Cry1A.105 and >25-fold for Cry2Ab2. Compared to the populations from the northeast Louisiana, the two populations collected from central Louisiana were more susceptible, with a resistance ratio of 15- to 35-fold to Cry1A.105 and 6- to 9-fold for Cry2Ab2. Dose responses of the two populations collected from Florida were similar as the two populations from central Louisiana, which exhibited a resistance ratio of 8- to 7-fold for Cry1A.105 and 9- to 15-fold to Cry2Ab2. The population collected from Georgia was also highly resistant to the Cry1A.105 protein with a resistance ratio of >909-fold, while it was relatively susceptible to the Cry2Ab2 protein with a resistance ratio of 5-fold.

The results of the laboratory bioassays validated that the field control problems of Cry1A.105/Cry2Ab2 Bt corn in northeast Louisiana was due to resistance development of CEW to the two Bt proteins in the plants. However, the variable susceptibility to the Cry1A.105/Cry2Ab2 proteins among the 12 field-collected populations suggests a mosaic distribution of the resistance in the south-east region of the U.S. Data of this study represent the first documentation of field resistance to Bt corn in a target pest species in the mid-south region of the U.S. Many factors might have contributed to the rapid development of field resistance to the pyramided Bt corn in CEW in the region. These factors included 1) both Bt corn and Bt cotton are planted in the region; 2) CEW is a cross-crop pest of corn and cotton as well as a cross-crop target of both Bt corn and Bt cotton in the region; 3) limited mode of action and similar Bt proteins have been used in both Bt corn and Bt cotton; 4) strong cross-resistance exists among Cry1 or Cry2 proteins expressed in the Bt corn and Bt cotton plants; and 5) different Bt

proteins in the pyramided Bt corn and Bt cotton have been introduced sequentially, but not at the same time.

The documentation of the field resistance to Cry1A.105/Cry2Ab2 Bt corn should have important implication for resistance management. Recently released pyramided Bt corn and Bt cotton varieties containing the Vip3A gene are still effective against the Cry1A.105/Cry2Ab2 resistant CEW populations. However, these pyramided Bt crops likely function as only the single-gene Bt crops and resistance could develop rapidly in the insect populations that are already resistant to the Cry1/Cry2 proteins. Additional mode of actions against CEW is urgently needed to ensure the long-term success of the transgenic Bt crops in U.S. south region. Further studies are also warranted to understand the detailed distribution of the resistance in the entire south region of the U.S. and to look for addition management methods to control the insect pests in both corn and cotton.

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## VITA

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