LSU Doctoral Dissertations

Graduate School

2014

Evaluation of Pyramided Bt Corn for Management of Corn Earworm and Fall Armyworm (Lepidoptera: Noctuidae)

Fei Yang
Louisiana State University and Agricultural and Mechanical College, fyang108@gmail.com

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

Part of the Entomology Commons

EVALUATION OF PYRAMIDED BT CORN FOR MANAGEMENT OF CORN
EARWORM AND FALL ARMYWORM (LEPIDOPTERA: NOCTUIDAE)

A Dissertation

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
Doctor of Philosophy

in

The Department of Entomology

by
Fei Yang
B.S., Yangzhou University, 2008
M.S., Yangzhou University, 2011
December 2014

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The fall armyworm (FAW), *Spodoptera frugiperda*, and corn earworm (CEW), *Helicoverpa zea*, are two target pests of pyramided Bt corn in the U.S.   This study determined the susceptibility of the two pests to pyramided Bt corn and evaluated if a 95:5% seed mixture of Bt and non-Bt corn was an appropriate approach for providing refuge populations of CEW for resistance management.   Firstly, susceptibility of 150 F_2 two-parent families to three common pyramided Bt corn traits was examined using 7-day leaf tissue bioassays and whole plant tests.   A few families survived the 7-day leaf tissue bioassays but progeny of the survivors from the leaf tissue bioassays could not survive in the whole plant tests, suggesting that the pyramided Bt corn products were effective against FAW. Secondly, occurrence and damage of CEW in three planting patterns of non-Bt and Bt corn plants containing SmartStax[TM] and Viptera[TM] 3111 traits were evaluated in the fields.   The studies showed that both Bt corn products were highly effective against CEW in both pure stand and mixed plantings.   Relative to pure non-Bt corn plantings, larval occurrence at the early stages (3-4 instars) in a mixed planting of 96% Bt and 4% of non-Bt corn were similar, but the larval development was delayed.   Finally, intensity of Bt protein contamination and its associated impacts on CEW populations in a mixed planting of 95% Bt and 5% non-Bt corn were assessed with the SmartStax[TM] traits.   The results of field and laboratory studies showed that cross-pollinations in the mixed planting caused majority (> 90%) of the refuge kernels to express ≥ one Bt protein, and the intensive Bt protein contamination in the refuge ears reduced neonate-to-adult survivorship of CEW to only 4.6%, a reduction of 88.1% relative to the larvae feeding on ears of pure non-Bt corn plantings.   The results

suggest that the 95:5% seed mixed approach cannot provide adequate refuge populations

for CEW.   Data generated from this study should provide useful information for

developing appropriate resistance management strategies for the sustainable use of the Bt

corn technology as a pest management tool.

CHAPTER 1. INTRODUCTION

1.1 Corn Biology and Production

1.1.1 Corn Biology

Corn, also called maize, *Zea mays L.*, is a plant belonging to the family Poaceae. The corn plants have separate male and female flowering organs.   The tassel is the male flowering structure producing pollens, while the ear shoot is the female structure.   Corn is a cross-pollinating crop in which most pollination results from pollen dispersed by wind and gravity (Bannert, 2006).   The growth of corn plants could be classified into two stages: vegetative (V) stage and reproductive (R) stage.   The vegetative stage lasts from corn emergence to tasselling, which takes about 60 days, depending on the temperature, moisture and other environmental conditions (Abendroth et al., 2011).   The reproductive period contains six stages from R1 (silking) to physiological maturity (R6), which lasts about another 60 days (Abendroth et al., 2011).

1.1.2 Corn Production

Corn is one of the most important economic crops widely planted throughout the world.   It is not only an important food for humans, but also an excellent source for animal feeding and raw materials for many industries, producing products like starch, oil, syrup and fuel ethanol.   According to the United States National Corn Growers Association (NCGA), the total global corn production is estimated >966.7 billion kilograms in 2013 (NCGA, 2014).   The United States is the largest corn producer in the world and contributes >353.6 billion kilograms, which accounted for 36.6% of the global corn production in 2013 (NASS, 2014a; NCGA, 2014).   Other major corn producing countries

1

including China, Brazil, Argentina, Ukraine, India, Mexico, Indonesia, France and South

Africa contributed a total of 44.6% corn production in 2013 (NASS, 2014a; NCGA, 2014).

In the last decade, the overall corn production had increased gradually from the year

2003 to 2013 in the United States.   In 2003, 78.6 million acres of corn were planted in the

United States (NASS, 2004).   By 2013, a total of 95.4 million acres were planted with

corn in the United States, which created a $61.3 billion production value for the agriculture

(NASS, 2014b).   In general, corn is widely planted within 41 states in the United States,

but dominated by west/north central and east central.   Illinois, Iowa and Nebraska are the

top three states in corn production in the United States (NASS, 2014b).   Corn also plays

an important role in the Louisiana agriculture and it is the second most widely planted field

crops following only soybean in the states.   During 2013, a total of 2,946 million

kilograms of corn was harvested from 680,000 acres in Louisiana with a total production

value of $591 million (NASS, 2014b).

1.2 Corn Insect Pests

Corn plants could be attacked and damaged by various insect pests such as thrips,

aphids, maggots, rootworms, corn borers, earworms, armyworms and others during

different stages of development.   However, the most important and destructive insect pests

on corn are generally classified into two categories, the above-ground lepidopteran species

and the under-ground coleopteran rootworms, *Diabrotica* spp (Difonzo and Collen, 2012).

The larvae of lepidopteran species cause damage by either consuming foliage, girdling of

the stalk, or feeding on the ears.   Major lepidopteran pests of corn in the United States

include the European corn borer, *Ostrinia nubilalis* (Hübner); southwestern corn borer,

Diatraea grandiosella (Dyar); stalk borer, *Papaipema nebris* (Guenee); sugarcane borer,

Diatraea saccharalis (Fabricius); fall armyworm, *Spodoptera frugiperda* (J.E. Smith); and

corn earworm, *Helicoverpa zea* (Boddie).   In the mid-southern regions of U.S., fall

armyworm, corn earworm, and a complex of sugarcane borer and southwestern corn borers

caused more serious damage to the corn plants (Siebert et al., 2012).   In contrast,

European corn borer and southwestern corn borer are the two predominant stalk borers in

the west/north central and east central (Mason, 1996; Williams et al., 1997).   The larvae of

corn rootworm species mainly attack and feed on the root tissues of corn plants, which

primarily include the southern corn rootworm, *Diabrotica undecimpunctata howardi*

(Barber); western corn rootworm, *Diabrotica virgifera virgifera* LeCont; and northern corn

rootworm, *Diabrotica barberi* Smith & Lawrence.   To manage and control the corn insect

pests, cultural practices like early planting and insecticides are widely applied in the corn

fields.   Currently, the most important tool for suppressing the populations of corn borers

and rootworms in the United States is the use of the transgenic corn products that express

Bacillus thuringiensis (Bt) proteins to kill the insects.

1.3 *Bacillus thuringiensis*

Bt is an aerobic, gram-positive, soil-dwelling bacterium, which can produce vegetative

insecticidal proteins (Vip) and crystalline (Cry) endotoxin, during the vegetative and

reproductive growth stages, respectively (Meeusen and Warren, 1989; Schnepf et al., 1998).

Most of these Bt proteins are specifically toxic to some insect pests, but are considered as

friendly compatible with the environments, humans and other organisms (e.g. pollinators,

parasitoids, fish).   Such characteristics make Bt an ideal candidate for biological

insecticide (Schnepf et al., 1998; Nester et al., 2002). Sprayable Bt formulations have been used for agricultural insect pest control for many years, but instability, narrow spectrum and incapacity to control cryptic species limited the wide application of these Bt sprays (Ferré and Van-Rie, 2002). An alternative way for effective use of Bt is to transfer the external Bt genes into plants, which make the genetically engineered plants express Bt toxins and kill the target insect pests (Gould, 1998).

Cry toxins are the most widely used Bt toxins in transgenic Bt plants. When ingested by insects, it needs a multistep process to transform the Cry proteins from a relatively inert crystalline protoxin form to a cytotoxic form (Schnepf et al., 1998). First, the environment of the midgut would promote crystal solubilization and the consequential release of protoxin. Second, cleavage sites on the protoxin are recognized and cut by insect proteases to produce active toxins that subsequently penetrate through the insect midgut peritrophic membrane and reach the midgut brush border membrane. Finally, these active toxins interact with specific receptors on the midgut epithelium, resulting in pore formation, swelling, lysis, and the eventual death of the insect (Pigott and Ellar, 2007; Knowles and Ellar, 1987).

1.4 Transgenic Bt Plants

Bt tobacco was the first transgenic plant expressing Bt toxins, which was developed in 1987. The Bt tobacco expressed Cry toxins for control of the tobacco hornworm, *Manduca sexta* (Linnaeus) (Vaeck et al., 1987; Barton et al., 1987). In 1995, potato plants producing Bt proteins were approved by the U.S. Environmental Protection Agency. In 1996, the first generation Bt corn, expressing a single Bt protein, was approved to be

commercialized in the United States.   Since then, the transgenic Bt corn, such as

YieldGard® expressing the Cry1Ab protein, were widely planted in the United States and

several other countries in the world.   In 2013, approximate 123.21 million acres of Bt corn

were planted in 16 countries for pest control in the world (James, 2013).   In the United

States alone, nearly 74.13 million acres of the field corn was planted to Bt corn, which

accounted for 76% of its total corn area in 2013 (NASS, 2013).

Before 2010, the first generation Bt corn planted in the United States expressed only a

single Bt gene against a target pest (Huang et al., 2006; US-EPA, 2002; 2004).   The major

first generation Bt corn products include YieldGard®, YieldGard® RW, Agrisure® CB/LL,

Agrisure® RW, Herculex® I, and Herculex® RW.   The YieldGard® and Agrisure® CB/LL

traits express Cry1Ab protein for control of lepidopteran pests such as European corn borer,

southwestern corn borer and sugarcane borer.   The Herculex® I product produces Cry1F

toxin for controlling the stalk borers and fall armyworm.   The YieldGard® RW, Agrisure®

RW and Herculex® RW traits contain Cry3Bb1, mCry3A and Cry34/35Ab1 proteins,

respectively, which are active against corn rootworms.   In 2010, the second generation

Bt corn, also called pyramided Bt corn, expressing two or more Bt proteins within the same

plant for control of a same target pest, became commercially available in the United States

and Canada (US-EPA, 2010a).   These pyramided products include Genuity® SmartStax™,

Genuity® VT Triple Pro™, Genuity® VT Double Pro™, Agrisure® Viptera™ 3111, and

Agrisure® Viptera™ 3110.   The Genuity® SmartStax™ trait expresses Cry 1A.105,

Cry2Ab2, and Cry1F proteins that are active against a variety of caterpillars (Lepidoptera),

as well as Cry34Ab1/Cry35Ab1 and Cry3Bb1 proteins that are active for suppressing corn

rootworms (Coleoptera) (Monsanto, 2012). Genuity[®] VT Triple Pro[TM] hybrids harbor

Cry 1A.105, Cry2Ab2 and Cry3Bb1, while Genuity[®] VT Double Pro[TM] possesses Cry

1A.105 and Cry2Ab2 (Monsanto, 2012). Agrisure[®] Viptera[TM] 3111contains Vip3A and

Cry1Ab for lepidoptera and mCry3A for rootworm and Agrisure® Viptera[TM] 3110 express

Vip3A and Cry1Ab proteins only (Syngenta, 2012; Burkness et al., 2010). All of these

pyramided products are expected to be more sustainable for controlling the target pests,

because they contain two or more different Bt genes with dissimilar modes of action

(Monsanto, 2012; Syngenta, 2012). The first generation Bt plants is expected to be

completely replaced by the pyramided Bt corn in the near future (Huang et al., 2014).

1.5 Bt Corn for Controlling Fall Armyworm and Corn Earworm

Several previous studies have evaluated the field efficacy of Bt corn against corn

earworm and fall armyworm (Burkness et al., 2002; 2010; Buntin et al., 2000; 2004; Buntin,

2008; Siebert et al., 2008). Buntin (2008) found the damage proportion of the single-gene

Cry1Ab corn by corn earworm in all trials exceeded 63% with $\geq$ 90% infested ears and the

single-gene Cry 1F corn was also not very effective against corn earworm. Fall

armyworm damages are frequently reported across the Southern region of the U.S. in

conventional non-Bt and single gene Cry1Ab corn varieties, especially when fields are

planted after the optimum seeding dates (Buntin, 2008). The initial reports of

field-derived resistance that resulted in control failures with Bt crops was the fall

armyworm population in Puerto Rico on Cry1F-expressing corn in 2006 (Matten et al.,

2008; Storer et al., 2010). These studies clearly suggested that neither the single-gene

YieldGard[®] (Cry1Ab) nor Herculex[®] I (Cry1F) technology produced a "high dose" against

fall armyworm and corn earworm, thus both species were not listed as targets for most of the first generation single-gene Bt corn products.

Compared to the first generation single-gene Bt corn, the second generation Bt corn technologies containing two or more Bt proteins are expected much more effective for both corn earworm and fall armyworm (Burkness et al., 2011). The overall field populations of corn stalk borers such as European corn borer in the United States has been decreased significantly since the use of the first generation single-gene Bt corn (Hutchison et al., 2010). Populations of the sugarcane borer in field corn in the U.S. mid-southern region also decreased considerably during the recent years (Huang et al., 2012). Nevertheless, both corn earworm and fall armyworm are considered as major targets of the second generation pyramided Bt corn in the United States, especially in the southern region.

1.5.1 Fall Armyworm

The fall armyworm is an economically important corn pest and native to the tropical regions of the Western Hemisphere from the United States to Argentina (Pashley et al. 1985; Pashley 1986; 1988). It is found throughout most of the United States east of the Rocky Mountains, but it does not overwinter in the Northeast because the pupae cannot survive where the ground freezes. It normally overwinters successfully in the United States only in southern Florida and southern Texas (Sparks, 1979; Buntin, 1986). Every year, populations migrate from these overwintered areas into various regions across the country including Louisiana. The life cycle of this pest consists of four stages (egg, larva, pupa and adult) and can be completed in about 30 days during the summer, but 60 days in the spring and autumn, and 80 to 90 days during the winter. Fall armyworm has a wide range

of > 80 host plants, including many major field crops such as corn, cotton, soybeans, sorghum, rice, alfalfa, and many vegetable plants. Larvae can cause damage by consuming foliage and also can burrow into the growing point (bud, whorl, etc.), destroying the growth potential of plants, or clipping the leaves. Control strategies of fall armyworm can rely on insecticides, cultural techniques, biological controls as well as Bt crop technologies. For example, insecticides are usually applied to protect plants against damage by fall armyworm during the silking stage. The most important cultural practice employed widely in southern states is early planting and/or use of early maturing varieties.

1.5.2 Corn Earworm

The corn earworm is one of the most destructive and difficult crop pests to control in agriculture. It is distributed throughout the United States except for Alaska (Capinera, 2000). This species is active throughout the year in tropical and subtropical climates, but becomes progressively more restricted to summer with increasing latitude. The number of generations is variable depending on the associated weather. For example, there has only one generation in Minnesota and western New York; two in northeastern states; three in northern California; four to five in Louisiana and southern California; and almost seven in southern Florida and southern Texas (Archer and Bynum, 1994; Capinera, 2000). Like fall armyworm, this pest also has the typically four stages to mature, including egg, larva, pupa and adult. Specifically, the egg can hatch in about three days after being deposited. The larva displays several instars, and six is normal but five, seven or eight is not uncommon. In addition, the larvae of this caterpillar come in a wide variety of colors, including shades of pink, green, brown and yellow, depending to some extent on the host

8

plants.   The whole period of larval stage lasts about 16 days.   The duration of the pupal

stage is about12 days during the summer.   Overall, the life cycle of this species can be

completed in about one month.   This pest can colonize > 200 host plants, most of which

are of economical importance, including lettuce, tomato, cucumber, squash, pumpkin, corn

(field corn and sweet corn) and cotton (Burkness et al., 2010).   Corn earworm is

considered to be the most costly crop pest in North America, causing extensive damages to

their host plants.   Damage to corn is primarily by larvae feeding in the terminals of young

plants and especially on the kernels of ear.   Management of this pest consists of the use of

insecticides, cultural practices, biological control, and Bt crop technologies.   Insecticides

are usually applied to foliage in a liquid formulation.   Trap cropping is often suggested as

a cultural practice for this insect because the high degree of preference by ovipositing

moths for corn.   Corn in the green silk stage can be used to lure moths from less preferred

crops.   Biological controls include application of nematodes and natural enemies.

In addition, cannibalism is also an important characteristic of this species.   Early

researches have indicated that cannibalism is a major impact on the population dynamics of

corn earworm in corn ears, leading up to 75% larval mortality (Barber, 1936; Stinner et al.,

1977).   Horner et al. (2003) also pointed cannibalistic behavior of corn earworm is a key

consideration for determining rate of adaption by this species to transgenic Bt plants.

Their results also showed that cannibalistic encounters could result in partially resistant

larvae feeding on nontoxic food, thus temporarily providing an escape from exposure to the

Bt endoprotein.   In addition, Chilcutt (2006) found that negative effects of Bt on larvae

could be compensated by increased cannibalism that increased larval survival to levels

comparable with larvae reared on non-Bt plants, implying that cannibalism may interfere with the use of Bt corn for control of corn earworm.

1.6 Bt Resistance

Widespread planting of Bt crops could potentially place a strong selection pressure on the pest populations, resulting in the development of resistance to Bt proteins (Gould, 1998; Tabashnik, 1994; Tabashnik et al., 2008; Ferré and Van-Rie, 2002; Bravo and Soberón, 2008). Laboratory selection experiments showed many insects have evolved resistance to Bt proteins. Indian meal moth, *Plodia interpunctella*, is the first studied case showing high resistance to Bt proteins in the laboratory (McGaughey, 1985). Other documentation of laboratory-selected resistance to Bt proteins include tobacco budworm, *Heliothis virescens* (Fabricius) (Gould et al., 1992; 1995; 1997), pink bollworm, *Pectinophora gossypiella* (Saunders) (Tabashnik et al., 2000; 2004), European corn borer (Huang et al., 1999; Siqueira et al., 2004; Pereira et al., 2008), sugarcane borer (Huang et al., 2006; 2007; 2008; 2009), and cotton bollworm, *Helicoverpa armigera* (Hübner) (Akhurst et al., 2003; Downes et al., 2007; Xu et al., 2005; 2009). In addition, the diamondback moth, *Plutella xylostella* (L.) was the first documented species evolved high levels of resistance to sprayable Bt formulations in the field in Hawaii (Tabashnik, 1994). Later, resistant populations of the cabbage looper, *Trichoplusia ni* (Hübner), to Bt sprays were reported in the greenhouses (Janmaat and Myers 2003; Kain et al., 2004). Moreover, field control failures or reduced efficacy of commercial transgenic Bt crops due to resistance development have been documented in at least four cases in the world (Huang et al., 2011). The first field resistance case was the resistance of fall armyworm to Cry1F in Bt corn in

Puerto Rico (Matten et al., 2008; Storer et al., 2010); the second was the African stem borer (*Busseola fusca*) resistance to Cry1Ab in Bt corn in South Africa (Van-Rensburg, 2007); the third was the resistance of pink bollworm to Cry1Ac in Bt cotton in west India (Dhurua and Gujar, 2011) and the fourth was the resistance of western corn rootworm to Cry3Bb1 in Bt corn in the United States (Gassmann et al., 2011).

The Bt pathogenesis pathway is very complex, and thus mechanisms of insects resistance to Bt toxins can be different. To date, numerous resistance mechanisms have been proposed in Bt-resistant insects, which include alterations of midgut digestive proteases, decreased peritrophic membrane permeability, heightened immune response, enhanced esterase production, reduced Cry toxin binding and mutations of ABC transporters (Schnepf et al., 1998; Bravo et al., 2011; Heckel et al., 2007; Tiewsiri and Wang, 2011; Ferré and Van-Rie, 2002; Tabashnik et al., 2011). In general, disruption of Bt toxin binding to midgut receptors is the most common mechanism of insect resistance. These most common midgut receptors include cadherin, aminopeptidase N (APN), alkaline phosphatase (ALP), and Glycolipid. For example, cadherin-mediated resistance has been identified in cotton bollworm, tobacco budworm, and pink bollworm (Xu et al., 2005; Gahan et al., 2001; Morin et al., 2003); aminopeptidase N–mediated resistance in beet armyworm, *Spodoptera exigua* and oriental leafworm*, Spodoptera litura* (Pigott and Ellar, 2007; Rajagopal et al., 2002); and alkaline phosphatase-mediated resistance in tobacco budworm, and fall armyworm (Jurat-Fuentes and Adang, 2004; Jurat-Fuentes et al., 2011). Thus, the resistance of mechanism is both species and Bt-toxin specific, and one resistance may be conferred by several mechanisms.

1.7 Bt Resistance Management

Evolution of resistance in target pest populations is the primary threat to the long term efficacy of Bt crops.   To delay resistance development of targeted pests and elongate the life-span of the Bt crop technology, U.S. Environmental Protection Agency (EPA) has deployed an insecticide resistance management (IRM) plan, also known as the "high-dose refuge" strategy (US-EPA, 1998).   The IRM strategy requires the Bt plants to produce a "high dose" of Bt toxins that can kill ≥ 95% resistant heterozygotes.   To ensure the success of the "high-dose refuge" IRM strategy, several assumptions should be met.   First, insect resistance to Bt toxins should be recessive such that both susceptible homozygotes (SS) and heterozygotes (RS) would be killed by the Bt crops.   Second, the initial resistance allele frequency in the target insect populations should be very rare (e.g. <0.001) (Andow and Alstad, 1998).   Third, the strategy requires farmers to grow a proportion of structured non-Bt crop refuges in the vicinity of Bt crops.   The purpose of planting non-Bt refuges is to sustain survival of susceptible pest populations such that these susceptible individuals develop without selection for resistance.   Ideally, rare resistant adults coming from Bt plants should mate with these susceptible pests from the refuge plants, and their resulting offspring are heterozygous that should be killed by the high dose Bt proteins in the plants (Ostile et al., 1997; US-EPA, 2001).

Another strategy for resistance management in Bt crops is the use of gene-pyramiding technology, which makes the transgenic plants contain two or more Bt proteins that are different in mode of actions but effective against the same target pests (Ghimire et al., 2011; Monsanto, 2012).   The use of these pyramided Bt corn hybrids is expected to be more

12

powerful to delay resistance development.   Because pyramided Bt crops have two or more

Bt proteins against a target pest, and once one of the proteins is out of control for the pests,

the remaining proteins can still take effect.

Although resistance evolution to pyramided Bt varieties should in general be slower,

resistance to pyramided Bt crop varieties is nonetheless driven by the same evolutionary

process as single Bt-protein varieties.   The main merit of pyramided Bt crop technologies

is the relatively low survival of heterozygous insects (Ives et al., 2011).   As mentioned

above, one of the key assumptions for the "high dose refuge" IRM strategy is the initial

resistance alleles of target pest populations in the field is very rare (e.g. <0.001) (Andow

and Alstad, 1998).   Monitoring of resistance evolution of field populations of the target

insect species as part of the current IRM plan is of great importance for the long-term

efficacy of Bt technologies.   In 2011, we established 150 two-parental family lines using

single-pair matings from three field populations of fall armyworm collected from Louisiana

and Florida.   Objectives 1 and 2 evaluated the susceptibility of these F_2 family lines of fall

armyworm to three common pyramided Bt corn traits, Genuity® VT Double Pro™ and

Genuity® SmartStax™ produced by Monsanto company and Agrisure® Viptera™ 3111

developed by Syngenta company.

Due to the compliance issue in the use of the structural refuge requirement for

resistance management from the growers, the U.S. EPA has approved to adopt a seed

mixture strategy (also called "refuge-in-the-bag" or "RIB") for providing refuge for

planting pyramided Bt corn hybrids in the U.S. Corn Belt where no cotton is planted

(Mallet and Porter, 1992; Matten et al., 2012; US-EPA, 2010a; Onstad et al., 2011).   For

the RIB strategy, a portion of non-Bt corn seeds are mixed with Bt corn seeds in each bag by seed industries prior to being sold to growers.   Growers just need to buy the premixed seeds and plant in their fields.   The attraction of seed mixture strategy for resistance management is that compliance issue associated with planting structured refuge among farmers could be eliminated (Onstad et al., 2011).   The RIB strategy has not been approved in the U.S. southern region where cotton is also planted (Matten et al., 2012). Several concerns prevent the use of the RIB strategy in the corn and cotton region.   For example, larval movement of the more Bt-tolerant pest species (e.g. corn earworm, fall armyworm) among Bt and non-Bt plants may create a favorable condition for resistance development (Mallet and Porter, 1992).   Larvae not receiving a lethal dose of Bt proteins that move off from Bt plants to non-Bt plants could increase heterozygote fitness and thus increase selection for resistance (Davis and Onstad, 2000).   Likewise, movement of susceptible larvae from non-Bt plants to Bt plants could reduce refuge efficacy by lowering the number of susceptible insects that will interbreed with potential resistance insects emerging from Bt plants.

More importantly, corn is a cross-pollinating crop.   In the field conditions, most pollen from the tassel settles within 20 to 50 feet and > 97% kernels in an ear are actually pollinated by other plants (Abendroth et al., 2011).   In the current "high-dose refuge" plan for insect resistance management, the toxin-free non-Bt plants are required to be planted next to the Bt corn varieties.   The purpose of non-Bt refuges is to conserve survival of susceptible pests.   However, gene flow due to pollen movement between Bt and non-Bt corn could disrupt this strategy, especially under the RIB scenario where the likelihood of

cross-pollination of non-Bt corn by Bt pollen is much higher compared to the structured refuges.   If this is the case, the cross-pollinated refuge ears would be contaminated by the Bt pollen resulting in Bt expressions in refuge ears that may kill susceptible individuals. In addition, pollen contamination also may increase the survival of heterozygous individuals relative to susceptible individuals.   Burkness et al. (2011) reported that non-Bt ears receiving Bt pollens were probably not providing the same level of non-selected moth production necessary for effectively mating with potentially Bt resistant moths emerging from Bt crops.   In addition, intermediate levels of Bt expression in ear kernels violates the important high-dose assumption necessary for Bt crop IRM (Burkness et al., 2011) and sub-lethal protein expression may speed up the selection for resistance (Onstad et al., 2011). Chilcutt and Tabashnik (2004) also indicated outcrossed and rogue plants could decrease effective refuge size for seed-feeding pests such as pink bollworm by increasing mortality of susceptible individuals.   Results of an Arizona study conducted by Heuberger et al. (2008) detected both Bt-outcrossed seeds and rogue Bt plants in refuge designs.   Corn earworms are seed-feeding pests of corn (Gore et al., 2005) and they also exhibit non-recessive resistance to Bt proteins (Burd et al., 2000).   Consequently, the heterozygous insects may have an advantage compared with susceptible individuals in contaminated refuges, which could accelerate the resistance evolution (Heuberger et al., 2008).

Argument over the effectiveness of RIB strategies for resistance management has been a hot topic for two decades and heated debates are still broadly existed (US-EPA, 2010; Chilcutt and Tabashnik, 2004; Burkness and Hutchison, 2012; Davis and Onstad, 2000;

Carroll et al., 2012; 2013).   The impact of pollen contamination on resistance

management has been evaluated in several studies but just for structured refuge

configuration and single-Bt gene corn products (Burkness et al., 2011; Chilcutt and

Tabashnik, 2004).   Yet, issues about pollen contamination in seed mixture with pyramided

Bt corn has not been evaluated against ear feeders such as corn earworm.   Several

mathematical models have shown that the seed mixture could be an effective insect

resistance management strategy for planting pyramided Bt corn (Carroll et al., 2012; Kang

et al., 2012).   However, scientific data to support the seed mixture strategy are still very

limited especially for ear-feeding insects (Alyokhin 2011; Kang et al., 2012; Onstad et al.

2011; US-EPA 2010a; 2010b; 2010c).   Therefore, research was needed to document insect

occurrence and damage in seed mixed plantings.   As mentioned above, corn earworm is a

major target species of pyramided Bt corn in the United States and its damage to corn is

primarily caused by larvae feeding on ear kernels.   Thus, the RIB-corn earworm system

provides an excellent model to study the effect of cross-pollination on refuge populations

of ear feeding species.   Objectives 3, 4, and 5 of this study were designed to evaluate the

Bt protein contamination in refuge ears, insect occurrence, larval development, and ear

injury of corn earworm in RIB plantings of non-Bt and pyramided Bt corn containing

Pyramided Bt corn.

1.8 Objectives

The specific objectives of this study contain:

1. Determine the susceptibility of fall armyworm to pyramided Bt corn hybrids containing

Genuity® VT Double Pro™ and Genuity® SmartStax™ traits;

2. Determine the susceptibility of fall armyworm to pyramided Bt corn hybrid containing Agrisure® Viptera™ 3111 traits;

3. Determine the occurrence, distribution, and ear damage of corn earworm in mixed plantings of non-Bt and pyramided Bt corn containing Genuity® SmartStax™ traits;

4. Determine Bt protein contamination and performance of Agrisure® Viptera™ 3111 Bt corn against corn earworm in seed mixed plantings;

5. Determine the intensity of Bt protein contamination in RIB plantings of non-Bt and pyramided Bt corn containing Genuity® SmartStax™ trait and the corresponding effect of the Bt protein contamination on survival, growth, and development of corn earworm.

1.9 References

Abendroth, L.J., Elmore, R.W., Boyer, M.J., Marlay, S.K., 2011. Corn growth and development. Ames, IA: PMR 1009, Iowa State University Extension, IA, US).

Akhurst, R.J., James, W., Bird, L.J., Beard, C., 2003. Resistance to the Cry1Ac delta-endotoxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). J. Econ. Entomol. 96, 1290-1299.

Alyokhin, A., 2011. Scant evidence supports EPA's pyramided Bt corn refuge size of 5%. Nat. Biotech. 29, 577-578.

Andow, D.A., Alstad, D.N., 1998. F2 screen for rare resistance alleles. J. Econ. Entomol. 91, 572–578.

Archer, T.L., Bynum, J.E.D., 1994. Corn earworm (Lepidoptera: Noctuidae) biology on food corn on the high plains. Environ. Entomol. 23, 343-348.

Bannert, M., 2006. Simulation of transgenic pollen dispersal by use of different grain colour maize. Ph.D. Dissertation, Swiss Federal Institute of Technology Zurich. Available at: http://www.agrisite.de/doc/ge_img/pollen-swiss.pdf.

Barber, G.W., 1936. The cannibalistic habits of the corn earworm. U.S. Dep. Agric. Technical Bulletin. 499, 1-19.

Barton, K.A., Whiteley, H.R., Yang, N.S., 1987. *Bacillus thuringiensis* §-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiol. 85, 1103-1109.

Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberón, M., 2011. *Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem. Mol. Biol. 41, 423-431.

Bravo, A., Soberón, M., 2008. How to cope with insect resistance to Bt toxins? Trends Biotechnol. 26, 573-579.

Buntin, G.D., 1986. A review of plant response to fall armyworm, *Spodoptera frugiperda* (J. E. Smith), injury in selected field and forage crops. Fla. Entomol. 69, 549-559.

Buntin, G.D., 2008. Corn expressing Cry1Ab or Cry1F endotoxin for fall armyworm and corn earworm (Lepidoptera: Noctuidae) management in field corn for grain production. Fla. Entomol. 91, 523-530.

Buntin, G.D., All, J.N., Lee, R.D., Wilson, D.M., 2004. Plant-incorporated *Bacillus thuringiensis* resistance for control of fall armyworm and corn earworm (Lepidoptera: Noctuidae) in corn. J. Econ. Entomol. 97, 1603-1611.

Buntin, G.D., Lee, R.D., Wilson, D.M., McPherson, R.M., 2000. Evaluation of YieldGard transgenic resistance for control of fall armyworm and corn earworm (Lepidoptera: Noctuidae) on corn. Fla. Entomol. 84, 37-42.

Burd, A.D., Bradley, J.R., Van-Duyn, J.W., Gould, F., 2000. Resistance of bollworm, *Helicoverpa zea*, to Cry1Ac toxin, pp. 923-926. In Proceedings of the Beltwide Cotton Conferences, 5-8 January 2000, San Antonio, TX, National Cotton Council of America, Memphis, TN.

Burkness, E.C., Dively, G., Patton, T., Morey, A.C., Hutchison, W.D., 2010. Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against Helicoverpa zea (Lepidoptera: Noctuidae) under field conditions. GM Crops 1, 1-7.

Burkness, E.C., Hutchison, W.D., 2012. Bt pollen dispersal and Bt kernel mosaics: integrity of non-Bt refugia for lepidopteran resistance management in maize. J. Econ. Entomol. 105, 1477-1870.

Burkness, E.C., Hutchison, W.D., Weinzierl, R.A., Wedberg, J.L., Wold, S., Shaw, J.T., 2002. Efficacy and risk efficiency of sweet corn hybrids expressing a *Bacillus thuringiensis* toxin for Lepidopteran pest management in the Midwestern US. Crop Protect. 21, 157-169.

Burkness, E.C., O'Rourke, P.K., Hutchison, W.D., 2011. Cross-pollination of nontransgenic corn ears with transgenic Bt corn: efficacy against lepidopteran pests and implications for resistance management. J. Econ. Entomol. 104, 1476-1479.

Capinera, J.L., 2000. Corn Earworm, *Helicoverpa* (=*Heliothis*) *zea* (Boddie) (Lepidoptera: Noctuidae). Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. EENY-145 (IN302).

Carroll, M.W., Head, G., Caprio, M., 2012. When and where a seed mix refuge makes sense for managing insect resistance to Bt plants. Crop Protect. 38, 74-79.

Carroll, M.W., Head, G., Caprio, M., Stork, L., 2013. Theoretical and empirical assessment of a seed mix refuge in corn for southwestern corn borer. Crop Protect. 49, 58-65.

Chilcutt, C.F., 2006. Cannibalism of *Helicoverpa zea* (Lepidoptera: Noctuidae) from *Bacillus thuringiensis* (Bt) transgenic corn versus non-Bt corn. J. Econ. Entomol. 99, 728-732.

Chilcutt, C.F., Tabashnik, B.E., 2004. Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maize. Proc. Natl. Acad. Sci. U.S.A. 101, 7526-7529.

Davis, P.M., Onstad, D.W., 2000. Seed mixtures as a resistance management strategy for European corn borers (Lepidoptera: Crambidae) infesting transgenic corn expressing Cry1Ab protein. J. Econ. Entomol. 93, 937-948.

Dhurua, S., Gujar, G.T., 2011. Field-evolved resistance to Bt protein Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) from India. Pest Manage. Sci. 67, 898-903.

Difonzo, C., Collen, E., 2012. Handy Bt trait table. Available at: http://www3.ag.purdue.edu/agry/PCPP/Documents/Entry%20forms/Handy_Bt_Trait_ Table.pdf.

Downes, S., Mahon, R., Olsen, K., 2007. Monitoring and adaptive resistance management in Australia for Bt-cotton: current status and future challenges. J. Invert Path. 95, 208-213.

Ferré, J., Van-Rie, J., 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Ann. Rev. Entomol. 47, 501-533.

Gahan, L.J., Gould, F., Heckel, D.G., 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science 293, 857-860.

Gassmann, A.J., Petzold-Maxwell, J.L., Keweshan, R.S., Dunbar, M.W., 2011. Field-evolved resistance to Bt maize by western corn rootworm. PloS One 6, e22629.

Ghimire, M.N., Huang, F., Leonard, R.B., Head, G.P., Yang, Y., 2011. Susceptibility of Cry1Ab-susceptible and -resistant sugarcane borer to transgenic corn plants containing single or pyramided *Bacillus thuringiensis* genes. Crop Protect. 30, 74-81.

Gore, J., Adamczyk, J.J., Blanco, C.A., 2005. Selective feeding of tobacco budworm and bollworm (Lepidoptera: Noctuidae) on meridic diet with different concentrations of *Bacillus thuringiensis* proteins. J. Econ. Entomol. 98, 88-94.

Gould, F., 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Ann. Rev. Entomol. 43, 701-726.

Gould, F., Andenson, A., Jones, A., Sumerford, D., Heckel, D.G., Lopez, L., Micinski, S., Leonard, B.R., Laster, M., 1997. Initial frequency of alleles for resistance to *Bacillus thuringiensis* toxins in field populations of Heliothis virescens. Pro. Nat. Acad. Sci. 94, 3519-3523.

Gould, F., Anderson, A., Reynolds, R., Bumgarner, L., Moar, W., 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 88, 1545-1559.

Gould, F., Martinez-Ramirez, A., Anderson, A., Ferré, J., Silva, F.J., Moar, W.J., 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. U.S.A. 89, 7986-7990.

Heckel, D.G., Gahan, L.J., Baxter, S.W., Zhao, J., Shelton, A.M., Gould, F., Tabashinik, B.E., 2007. The diversity of Bt resistance genes in species of Lepidoptera. J. Invertebr. Pathol. 95, 192-197.

Heuberger, S., Ellers-Kirk, C., Yafuso, C., Gassmann, A.J., Tabashnik, B.E., Dennehy, T.J., Carriere, Y., 2008. Effects of refuge contamination by transgenes on Bt resistance in pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 101, 504-514.

Horner, T.A., Dively, G.P., 2003. Effects of MON810 Bt field corn on *Helicoverpa zea* (Lepidoptera: Noctuidae) cannibalism and its implication to resistance developement. J. Econ. Entomol. 96, 931-934.

Huang, F., Andow, D.A., Buschman, L.L., 2011. Success of the high dose/refuge resistance management strategy after 15 years of Bt crops in North America. Entomol. Exp. Appl. 140, 1-16.

Huang, F., Ghimire, M.N., Leonard, B.R., Daves, C., Levy, R., Baldwin, J., 2012. Extended monitoring of resistance to *Bacillus thuringiensis* Cry1Ab maize in *Diatraea saccharalis* (Lepidoptera: Crambidae). GM Crops 3, 245-254.

Huang, F., Leonard, B.R., 2008. Detection and monitoring of Bacillus thuringiensis resistance alleles in the sugarcane borer, *Diatraea saccharalis* (F.) pp 1-17. In "Genetically Modified Plants: New Research Trends", (eds) by T.V. Wolf and J.P. Koch. Nova Science Publishers, Hauppauge, NY.

Huang, F., Leonard, B.R., Gable, R.H., 2006. Comparative susceptibility of European corn borer, southwestern corn borer, and sugarcane borer (Lepidoptera: Crambidae) to Cry1Ab protein in a commercial *Bacillus thuringiensis* corn hybrid. J. Econ. Entomol. 99, 194-202.

Huang, F., Leonard, B.R., Wu, X., 2007. Resistance of sugarcane borer to *Bacillus thuringiensis* Cry1Ab toxin. Entomol. Exp. Appl. 124, 117-123.

Huang, F., Parker, R., Leonard, B.R., Yang, Y., Liu, J., 2009. Frequency of resistance alleles to *Bacillus thuringiensis*-corn in Texas populations of the sugarcane borer, *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae). Crop Protect. 28, 174-180.

Huang, F., Qureshi, J.A., Meagher-Jr, R.L., Reisig, D.D., Head, G.P., Andow, D.A., Ni, X., Kerns, D.L., Buntin, D.G., Niu,Y., Yang, F., Dangal, V., 2014. Cry1F resistance in fall armyworm: single gene versus pyramided Bt maize. PLoS One (In Press).

Huang, F., Zhu, K.Y., Buschman, L.L., Higgins, R.A., 1999. Comparison of midgut proteinases in *Bacillus thuringiensis* susceptible and - resistant European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). Pest Biochem. Physiol. 65, 132-139.

Hutchison, W.D., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S.J., Abrahamson, M., Hamilton, K.L., Steffey, K.L., Gray, M.E., Hellmich, R.L., Kaster, L.V., Hunt, T.E., Wright, R.J., Pecinovsky, K., Rabaey, T.L., Flood, B.R., Raun, E.S., 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science 330, 222-225.

Ives, A.R., Glaum, P.R., Ziebarth, N.L., Andow, D.A., 2011. The evolution of resistance to two-toxin pyramided transgenic crops. Ecol. Appl. 21, 503-515.

James, C., 2013. Global status of commercialized biotech/GM crops: 2013. ISAAA Brief No. 45. ISAAA: Ithaca, NY, USA.

Janmaat, A.F., Myers, J.. 2003. Rapid evolution and the cost of resistance to *Bacillus thuringiensis* in greenhouse populations of cabbage looper, *Trichoplusia ni*. Proc. R. Soc. Lond. B. 270, 2263-2270.

Jurat-Fuentes, J.L., Adang, M.J., 2004. Characterization of a Cry1Ac receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. Eur. J. Biochem. 271, 3127-3135.

Jurat-Fuentes, J.L., Karumbaiah, L., Jakka, S.R.K., Ning, C., Liu, C., Wu, K., Jackson, J., Gould, F., Blanco, C., Portilla, M., Perera, O., Adang, M., 2011. Reduced levels of membrane-bound alkaline phosphatase are common to Lepidopteran strains resistant to Cry Toxins from *Bacillus thuringiensis*. PLoS One 6, e17606.

Kain, W.C., Zhao, J., Janmaat, A.F., Myers, J., Shelton, A.M., Wang, P., 2004. Inheritance of Resistance to Bacillus thuringiensis Cry1Ac Toxin in a Greenhouse-Derived Strain of Cabbage Looper (Lepidoptera: Noctuidae). J. Econ. Entomol. 97, 2073-2078.

Kang, J., Onstad, D.W., Hellmich, R.L., Moser, S.E., Hutchison, W.D., Prasifka, J.R., 2012. Modeling the impact of cross-pollination and low toxin expression in corn kernels on adaptation of European corn borer (Lepidoptera: Crambidae) to transgenic insecticidal corn. Environ. Entomol. 41, 200-211.

Knowles, B.H., Ellar, D.J., 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* endotoxins with different insect specificity. Biochim. Biophys. Acta 924, 507-518.

Mallet, J., Porter, P., 1992. Preventing insect adaptation to insect-resistant crops: are seed mixtures or refugia the best strategy? Proc. R. Soc. Lond. B. 250,165-169.

Mason, C.E., 1996. European corn borer: ecology and management. Iowa State University. NCR327.

Matten, S.R., Frederick, R.J., Reynolds, A.H., 2012. United States Environmental Protection Agency insect resistance management programs for plant-incorporated protectants and use of simulation modeling. Regulation of agricultural biotechnology: The United States and Canada, eds Wozniak CA, McHughen A (Springer), pp 175-267.

Matten, S.R., Head, G.P., Quemada, H.D., 2008. How governmental regulation can help or hinder the integration of Bt crops into IPM programs. Integration of Insect-Resistant Genetically Modified Crops within IPM Programs, eds Romeis J, Shelton AM, Kennedy GG (Springer, New York), pp. 27-39.

McGaughey, W.H., 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science 229, 193-195.

Meeusen, R.L., Warren, G., 1989. Insect control with genetically engineered crops. Ann. Rev. Entomol. 34, 373-381.

Monsanto, 2012. IRM grower guide: insect resistance management for U.S. corn and cotton-growing areas. Available at: http://www.monsanto.com/products/pages/insect-resistance-management.aspx.

Morin, S., Biggs, R.W., Sisterson, M.S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L.J., Heckel, D.G., Carrière, Y., Dennehy, T.J., Brown, J.K., Tabashnik, B.E., 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. U.S.A. 100, 5004-5009.

NASS (National Agricultural Statistics Service), 2004. Acreage. Available at: http://usda.mannlib.cornell.edu/usda/nass/Acre//2000s/2004/Acre-06-30-2004.pdf

NASS (National Agricultural Statistics Service), 2013. Acreage. USDA, Washington DC. USA. Available at: http://usda01.library.cornell.edu/usda/current/Acre/Acre-06-28-2013.pdf.

NASS (National Agricultural Statistics Service), 2014a. World agricultural supply and demand estimates. Available at: http://www.usda.gov/oce/commodity/wasde/latest.pdf

NASS (National Agricultural Statistics Service), 2014b. Acreage. Available at: http://usda.mannlib.cornell.edu/usda/nass/Acre//2010s/2014/Acre-06-30-2014.pdf

NCGA (National Corn Growers Association), 2014. World of corn 2014. Available at: http://www.ncga.com/upload/files/documents/pdf/woc-2014.pdf

Nester, E.W., Thomashow, L.S., Metz, M., Gordon, M., 2002. 100 Years of *Bacillus thuringiensis*: A critical scientific assessment. American Academy of Microbiology, Washington, DC.

Onstad, D.W., Mitchell, P.D., Hurley, T.M., Lundgren, J.G., Porter, R.P., Krupke, C.H., Spencer, J.L., DiFonzo, C.D., Baute, T.S., Hellmich, R.L., Buschman, L.L., Hutchison, W.D., Tooker, J.F., 2011. Seeds of change: corn seed mixtures for resistance management and IPM. J. Econ. Entomol. 104, 343-352.

Ostlie, K.R., Hutchison, W.D., Hellmich, R.L., 1997. Bt-Corn & European Corn Borer, Long Term Success through Resistance Management. North Central Region Extension Publication 602. University of Minnesota, St. Paul, MN, USA.

Pashley, D.P., 1986. Host associated genetic differentiation in fall armyworm (Lepidoptera: Noctuidae): a sibling species complex. Ann. Entomol. Soc. Amer. 79, 898-904.

Pashley, D.P., 1988. Quantitative genetics, development, and physiological adaptation in host strains of fall armyworm. Evolution 42, 93-102.

Pashley, D.P., Johnson, S.J., Sparks, A.N., 1985. Genetic population structure of migratory moths: the fall armyworm (Lepidoptera: Noctuidae). Ann. Entomol. Soc. Amer. 78, 756-762.

Pereira, E.J.J., Storer, N.P., Siegfried, B.D., 2008. Inheritance of Cry1F resistance in laboratory- selected European corn borer and its survival on transgenic corn expressing the Cry1F toxin. Bull. Entomol. Res. 98, 621-629.

Pigott, C.R., Ellar, D.J., 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiol. Mol. Biol. Rev. 71, 255-281.

Rajagopal, R., Sivakumar, S., Agrawal, N., Malhotra, P.M., Bhatnagar, R.K., 2002. Silencing of midgut aminopeptidase N of *Spodoptera litura* by double stranded RNA establishes its role as *Bacillus thuringiensis* receptor. J. Biol. Chem. 277, 46849-46851.

Schnepf, E., Crickmore, N., Van-Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 775-806.

Siebert, M.W., Nolting, S.P., Hendrix, W., Dhavala, S., Craig, C., Leonard, B.R., Stewart, S.D., All, J., Musser, F.R., Buntin, G.D., Samuel, L., 2012. Evaluation of corn hybrids expressing Cry1F, Cry1A.105, Cry2Ab2, Cry34Ab1/Cry35Ab1, and Cry3Bb1 against southern United States insect pests. J. Econ. Entomol. 105, 1825-1834.

Siebert, M.W., Tindall, K.V., Leonard, B.R., Van-Duyn, J.W., Babcock, J.M., 2008. Evaluation of corn hybrids expressing Cry1F (Herculex® I insect protection) against fall armyworm (Lepidoptera: Noctuidae) in the Southern United States. J. Entomol. Sci. 43, 41-51.

Siqueira, H.A.A., Spencer, T., Moellenbeck, D.J., Siegfried, B.D., 2004. Cross-resistance of Cry1Ab-selected *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) to *Bacillus thuringiensis* delta-endotoxins. J. Econ. Entomol. 97, 1049-1057.

Sparks, A.N., 1979. A review of the biology of the fall armyworm. Fla.Entomol. 62, 82-87.

Stinner, R.E., Jones, J.W., Tuttle, C., Caron, R.E., 1977. Population mortality and cyclicity as affected by intraspecific competition. Can. Entomol.109, 879-890.

Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W., Huckaba, R.M., 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 103, 1031-1038.

Syngenta, 2012. Corn trait Information: Agrisure Viptera™ 3111. Available at: http://www.syngenta.com/country/us/en/Seeds/Traits/CornTraits/Pages/content_author ing_Agrisurevip3111.aspx.

Tabashnik, B.E., 1994. Evolution of resistance to Bacillus thuringiensis. Ann. Rev. Entomol. 39, 47-79.

Tabashnik, B.E., Gassmann, A.J., Crowder, D.W., Carrière, Y., 2008. Insect resistance to Bt crops: evidence versus theory. Nat. Biotech. 26, 199-202.

Tabashnik, B.E., Huang, F., Ghimire, M.N., Leonard, B.R., Siegfried, B.D., Rangasamy, M., Yang, Y., Wu, Y., Gahan, L.J., Heckel, D.G., Bravo, A., Soberón, M., 2011. Efficacy of genetically modified Bt toxins against insects with different genetic mechanisms of resistance. Nat. Biotechnol. 29, 1128-1131.

Tabashnik, B.E., Liu, Y.B., De Maagd, R.A., Dennehy, T.J., 2000. Cross-resistance of of pink bollworm to *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. 66, 4582-4584.

Tabashnik, B.E., Liu, Y.B., Unnithan, D.C., Carrière, Y., Dennehy, T.J., Morin, S., 2004. Shared genetic basis of resistance to Bt toxin Cry1Ac in independent strains of pink bollworm. J. Econ. Entomol. 97, 721-726.

Tiewsiri, K., Wang, P., 2011. Differential alteration of two aminopeptidases N associated with resistance to *Bacillus thuringiensis* toxin Cry1Ac in cabbage looper. Proc. Natl. Acad. Sci. U.S.A. 108, 14037-14024.

US-EPA (United States Environmental Protection Agency), 2001. Biopesticides Registration Action Document-*Bacillus thuringiensis* Plant-Incorporated Protectants. E: Benefit Assessment. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/Bt_brad2/5-benefits.pdf

US-EPA (United States Environmental Protection Agency), 2010a. Terms and conditions for Bt corn registrations 30 Sept 2010. Office of Pesticide Programs, Washington, DC. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/bt-corn-terms-conditions.pdf.

US-EPA (United States Environmental Protection Agency), 2010b. Biopesticides registration action document: Cry1Ab and Cry1F Bt plant-incorporated protectants. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/cry1f-cry1ab-brad.pdf

US-EPA (United States Environmental Protection Agency), 2010c. Biopesticides registration action document: Bacillus thuringiensis Cry1A.105 and Cry2Ab2 insecticidal proteins and the genetic material necessary for their production in corn. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/mon-89034-brad.pdf

US-EPA (US Environmental Protection Agency), 1998. Report of subpanel on *Bacillus thuringiensis* (Bt) plant-pesticides and resistance management. Available at: http://www.epa.gov/scipoly/sap/meetings/1998/february/finalfeb.pdf

US-EPA (US Environmental Protection Agency), 2002. *Bacillus thuringiensis* Cry2Ab2 protein and the genetic material necessary for its production in cotton (006487) fact sheet. Available at: http://www.epa.gov/oppbppd1/biopesticides/ingredients/factsheets/factsheet_006487.htm

US-EPA (US Environmental Protection Agency), 2004. *Bacillus thuringiensis* Cry1F corn biopesticide registration action document (BRAD). Available at: http://www.epa.gov/oppbppd1/biopesticides/ingredients/tech_docs/brad_006481.pdf

Vaeck, M., Reynaerts, A., Höfte, H., Jansens, S., Beuckeleer, M.D., Dean, C., Zabeau, M., Montagu, M.V., Leemans, J., 1987. Transgenic plants protected from insect attack. Nature 328, 33-37.

Van-Rensburg, J.B.J., 2007. First report of field resistance by stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. S. Afr. J. Plant and Soil 24,147–151.

Williams, W.P., Sagers, J.B., Hanten, J.A., Davis, F.M., Buckley, P.M., 1997. Transgenic corn evaluated for resistance to fall armyworm and southwestern corn borer. Crop Sci. 37, 957-962.

Xu, X., Yu, L., Wu, Y., 2005. Disruption of a cadherin gene associated with resistance to Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. Appl. Environ. Microbiol. 21, 948-954.

Xu, Z., Liu, F., Chen, J., Huang, F., Andow, D.A., Shen, J., Zhu, Y. C., 2009. Using F2 screen to monitor resistance allele frequency to Bt cotton in field populations of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Pest Manag. Sci. 65, 391-397.

CHAPTER 2. SUSCEPTIBILITY OF LOUISIANA AND FLORIDA POPULATIONS OF *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTUIDAE) TO PYRAMIDED BT CORN CONTAINING GENUITY® VT DOUBLE PRO™ AND SMARTSTAX™ TRAITS[1]

2.1 Introduction

Since its first commercialization in 1996, transgenic corn expressing *Bacillus thuringiensis* (Bt) proteins has been widely planted worldwide, especially in the United States and Canada (James, 2013). In general, these Bt corn hybrids are effective in suppressing two classes of corn pests: above-ground Lepidoptera such as the European corn borer, *Ostrinia nubilalis* (Hübner) (Crambidae), and southwestern corn borer, *Diatraea grandiosella* Dyar (Crambidae); and below-ground Coleoptera such as the western corn rootworm *Diabrotica virgifera virgifera* Leconte (Chrysomelidae) and northern corn rootworm *Diabrotica barberi* Smith & Lawrence. Until now, transgenic Bt technology for corn can be classified into two generations (Buntin and Flanders, 2012; Huang et al., 2012). First generation transgenic corn hybrids express a single Bt gene for controlling a target species. These single-gene Bt corn products are very efficient in controlling the major stalk borer species, but most have only limited efficacy for suppressing fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Noctuidae) (Adamczyk and Mahaffey, 2008; Huang et al., 2011). Fall armyworm is an important corn pest in the Western Hemisphere from the United States to Argentina (Pashley et al., 1985; Pashley, 1986; 1988). Studies have shown that all first generation single-gene Bt corn products do not produce a "high dose" for fall armyworm (Chilcutt et al., 2007; Adamczyk and Mahaffey, 2008; Hardke et al., 2011; Huang et al., 2011). As a result, fall armyworm is

---

[1] Reprinted with permission by the Florida Entomologist

excluded from the target list for single-gene Bt corn products except for Herculex®I which expresses the Cry1F protein (US-EPA, 2001; 2005).   Unfortunately, with the extensive use of Herculex®I corn in Puerto Rico, field resistance of fall armyworm to Cry1F corn was observed in 2006 (Matten et al., 2008; Storer et al., 2010).   The Cry1F resistance in fall armyworm was shown to be autosomally inherited and recessive (Storer et al., 2010; 2012a).   To broaden the target spectrum and delay resistance development, a gene pyramiding strategy has been used to develop transgenic Bt corn containing two or more Bt proteins that are dissimilar in mode of action but effective against the same target pests (Monsanto, 2012).   This strategy relies on the expression of Bt proteins with different modes of action in a pyramided product.   Therefore, the likelihood for evolution of resistance to a pyramided product is expected to be lower than against single trait products (Monsanto, 2012).   These second generation pyramided Bt corn hybrids are more effective in controlling fall armyworm (Burkness et al., 2010; Niu et al., 2013).   Consequently, fall armyworm is listed as a target species for all currently commercialized pyramided Bt corn products in the United States (Monsanto, 2012; Syngenta, 2012).

Pyramided Bt corn products also require certain necessary actions to maintain their durability in the marketplace.   In this regard, a major potential threat is the evolution of resistance in target pests.   In 2011, we established 149 two-parent families using single-pair matings from three field populations of fall armyworm collected from Louisiana and Florida.   The objective of this study was to examine the susceptibility of these families to two major pyramided Bt corn traits, Genuity® VT Double Pro™ and Genuity® SmartStax™.

2.2 Materials and Methods

2.2.1 Insect Collection and Rearing

Feral larvae of fall armyworm were collected during 2011 from sorghum fields in Franklin and Rapides parishes in Louisiana and from sweet corn fields in Collier County in Florida. In each location, larvae were sampled at two different times with a 1- to 2-wk interval between the two samplings in each location. All field-collected larvae were reared individually on a meridic diet (WARD'S Stonefly Heliothis diet, Rochester, New York) until pupal stage as described in Niu et al. (2013).

2.2.2 Use of Single Pairs to Establish Two-Parent Family Lines

Newly emerged virgin male and female moths of fall armyworm derived from the field-collected individuals were paired in 2- or 3.8-L paper containers (Huhtamaki Foodservice, De Soto, Kansas) containing ~100g of vermiculite (Sun Gro, Pine Bluff, Arkansas). Adult containers were placed in environmental chambers maintained at 28 $^{\circ}$C, > 90% RH, and 14:10 h L: D for adult emergence, mating, and oviposition. Progeny (F_1) produced from each pair were considered as a two-parent family and was reared individually in the 30-mL cups containing meridic diet as mentioned above (Niu et al., 2013). Fifty-to-eighty F_1 adults of each family were sib-mated in 3.8 L cardboard cartons (Neptune Paper Products, Newark, New Jersey) to produce F_2 generation for each two-parent family.

2.2.3 Source of Plant Materials

Leaf tissue of two pyramided corn hybrids, DKC 64-04 containing Genuity$^{®}$ VT Double Pro^{TM} traits and DKC 61-21 containing Genuity$^{®}$ SmartStax^{TM} traits (Monsanto, St

Louis, Missouri), was used in examination of the susceptibility of fall armyworm (Table 2.1). Genuity® VT Double Pro™ (MON89034) corn contains the Cry1A.105 and Cry2Ab2 proteins for controlling above-ground lepidopteran species including fall armyworm. Genuity® SmartStax™ expresses six Bt proteins including the two Bt proteins in Genuity® VT Double Pro™ together with Cry1F (TC1507) targeting lepidopteran pests and Cry3Bb1 (MON88017) and Cry34/35Ab1 (DAS-59122) for controlling underground rootworms, *Diabrotica* spp. In addition, a genetically closely related non-Bt corn hybrid, DKC 61-22 (Monsanto, St Louis, Missouri), was used to establish the baseline survival of fall armyworm. Both Bt and non-Bt corn hybrids were planted in 18.9 L pots each containing approximately 5 kg of a standard potting soil mixture in a greenhouse located at the Louisiana State University Agricultural Center in Baton Rouge, Louisiana as described in Wu et al. (2007). Expression of Cry1A.105, Cry2Ab2, and Cry1F proteins in plants was also confirmed using ELISA-based assays (EnviroLogix, Quantiplate™ kits, Portland, Maine).

Table 2.1. Hybrids used in evaluation of susceptibility of *Spodoptera frugiperda* family lines to Bt corn.

Corn trait	Corn hybrid	Event	Bt genes	Major target pest
Non-Bt	DKC 61-22	--	--	--
Genuity® VT Double Pro™	DKC 64-04	MON89034	Cry1A.105, Cry2Ab2	lepidoptera
Genuity® SmartStax™	DKC 61-21	MON89034+ MON88017+TC1507 + DAS-59122	Cry1A.105, Cry2Ab, Cry1F, Cry3Bb1, Cry34/35Ab	lepidoptera & rootworms

2.2.4 Leaf Tissue Bioassays

Bioassays for examining susceptibility to Genuity® VT Double Pro™ and Genuity®
SmartStax™ corn in fall armyworm were performed in 32-well trays (Bio-Smart-32, C-D
International, Pitman, New Jersey) containing corn leaf tissues of V4-V10 stages of
greenhouse grown corn plants.   Based on our preliminary study involved in consideration of
both larval cannibalism effect and labor intensity needed in the bioassay, four individuals of
fall armyworm in each well were considered to be an appropriate number used in the leaf
tissue bioassay to minimize larval cannibalism.   For each insect family and Bt corn hybrid, a
total of 96 F_2 neonates were assayed in 24 wells (4 neonates/well) each containing 2-4 pieces
of approximately 3-cm fresh leaf tissue as described in Niu et al. (2013).   The bioassay trays
were placed in growth chambers maintained at 28°C, ~50% RH and 16:8 h L:D. Leaf tissue
was replaced every 3 days.   The number of live larvae, larval stages (small larvae: $\leq$ 2nd
instar and large larvae: $\geq$ 3rd instar), and larval body mass of small (1st and 2nd instars) and
large larvae ($\geq$3rd instars) were recorded at 7 d after inoculation.

2.2.5 Baseline Survival

Baseline survival of a Bt-susceptible strain (Bt-SS) of fall armyworm on leaf tissue of
non-Bt and the two Bt corn hybrids was determined using the same method of the leaf tissue
bioassays described above.   Bt-SS was established from larvae collected from corn fields in
Hendry County, Florida, USA during 2011, and it has been documented to be susceptible to
Purified Cy1F protein as well as Genuity® VT Double Pro™ and Genuity® SmartStax™ (Niu
et al., 2013).   In addition, larval survival of seven F_2 families of fall armyworm established
from the field collections in Louisiana and Florida was also examined on corn leaf tissue of

four non-Bt corn hybrids [Agrisure[®] NK N78N-GT (Syngenta, Minnetonka, Minnesota), Pioneer 31G66 (Pioneer Hi-Bred, Johnston, Iowa), DKC 67-86 and DKC 61-22 (Monsanto, St. Louis, Missouri)] using the same method as used for assaying the Bt-SS strain.   For statistical analysis, mortality data of the F_2 families collected from a state were combined across the non-Bt corn hybrids.   Mortality data were transformed with arcsine $(x)^{0.5}$, and then subjected to a one-way analysis of variance (ANOVA).   Differences in larval mortality among the three sources (Louisiana, Florida, and Bt-SS) of fall armyworm were separated with LSD tests at $\alpha = 0.05$ level (SAS Institute, 2010).

Furthermore, larval mortality of the Bt-SS and seven F_2 families was also individually assayed on a meridic diet (Ward's Stonefly Heliothis diet, Rochester, New York) as described in Niu et al. (2013).   A total of seven independent bioassays were conducted for Bt-SS strain during the period of this study, while there were six and one bioassays for the F_2 families collected from Florida and Louisiana, respectively.   In each bioassay, approximately 1 g of diet was placed into each cell of the 128-cell trays (C-D International, Pitman, New Jersey). One neonate (< 24 h) was released on the diet in each cell.   The bioassay trays were placed in growth chambers maintained at 28 $^{°}$C, ~50% RH, and 16:8 h L: D.   Larval mortality was recorded on the 7th day after inoculation.   In each bioassay, there were four replications with 32 larvae in each replication.   As described in the leaf tissue bioassays, mortality data observed in the diet bioassays were transformed with arcsine $(x)^{0.5}$, and then subjected to a one-way ANOVA.   Differences in larval mortality among the three insect sources were separated with LSD tests at $\alpha = 0.05$ level (SAS Institute, 2010).

2.2.6 Re-evaluation of Tolerant Strains

Nine F_2 families survived well in the leaf tissue bioassays (see results).   Based on the baseline survivorship of the susceptible population of fall armyworm on non-Bt plant leaf tissue, these families that had ≥ 3 large live larvae with a body mass of ≥ 30 mg of all large larvae after seven days in the leaf tissue bioassays were considered to be potentially tolerant to the Bt corn traits.   These same criteria were used to define the F_2 families possessing major resistance alleles to Cry1F corn plants (Huang et al., unpublished data).   Laboratory strains of the potentially tolerant families were established from the survivors in the leaf tissue bioassays.   These laboratory strains were then re-evaluated for larval survival on Bt leaf tissue in the laboratory and whole Bt corn plants in the greenhouse.   A total of two leaf tissue tests and two whole plant trials were conducted for each family.   The leaf tissue tests were carried out in the same way as described in the bioassays with the F_2 families described above.   In the whole plant tests, five neonates of a family were inoculated into the whorl of each plant at V9-V10 stages of Bt and non-Bt corn hybrids.   A total of 50 neonates of each tolerant family were infested to ten Bt plants within five pots in each test.   Bt plants were confirmed for Bt protein expression with the ELISA-based assays (EnviroLogix, Quantiplate^{TM} Kits, Portland, Maine).   Similarly, for the test on non-Bt corn plants, a total of 60 neonates of a tolerant family were infested on 12 non-Bt plants (five neonates/ plant). Leaf injury ratings were recorded based on Davis' 1(no injury) to 9 (heavy injury) scale (Davis et al., 1992) after 7 and 13 days, respectively, and the number of live larvae per plant was checked after 13 days.   In addition, larval survival and leaf injury of Bt-SS strain were also evaluated on whole plants of the non-Bt (DKC 61-22) and the two Bt corn hybrids in the

greenhouse to verify the insecticidal activity of the two Bt corn hybrids.   In the tests with

Bt-SS, a total of 48 neonates were infested in 16 plants (three neonates/ plant) of each hybrid

at the V6-V8 plant stages.   Insect survival and leaf injury ratings were recorded 15 days

after release of neonates.

2.3 Results

2.3.1 Baseline Survivorship on Corn Leaf Tissue and Meridic Diet

Larval survivorship rate of the Bt susceptible population of fall armyworm was $59.2 \pm$

1.8% on non-Bt corn leaf tissue after 7 days, while it was zero on leaf tissues of both

Genuity® VT Double Pro™ and Genuity® SmartStax™ corn, suggesting leaf tissue of both

pyramided Bt corn products expressed a sufficient level of Bt proteins to kill susceptible fall

armyworm and thus could be used for identifying individuals that were tolerant to the two Bt

corn products.   The effect of insect sources (Bt-SS, Louisiana and Florida) on larval survival

on non-Bt corn leaf tissue was not significant ($F = 0.82$; df = 2, 69; $P = 0.4464$).   The 7-day

larval survivorship rates of the two-parent families collected from Louisiana and Florida were

$57.3 \pm 2.2\%$ and $61.4 \pm 2.4\%$, respectively, which was not significantly different ($P > 0.05$)

compared to the mortality observed for Bt-SS.   Effect of insect sources on larval mortality

on meridic diet was also not significant ($F = 1.39$; df = 2, 53; $P = 0.2585$).   Larvae of Bt-SS

and the families derived from field collections survived well on the meridic diet with a 7-day

mortality of $10.9 \pm 1.7\%$ for Bt-SS, $3.2 \pm 1.3\%$ for the families collected from Louisiana, and

$9.7 \pm 1.4\%$ from Florida.

2.3.2 Susceptibility of Louisiana Populations of Fall Armyworm to Genuity$^®$ VT Double Pro^{TM} and Genuity$^®$ SmartStax^{TM}

A total of 48 F_2 families (or 96 feral individuals) and 29 F_2 families (or 58 feral individuals) of fall armyworm were established from larvae collected from Franklin and Rapides parishes in Louisiana, respectively.   All of these F_2 families were examined for susceptibility on leaf tissue of Genuity$^®$ VT Double Pro^{TM} and Genuity$^®$ SmartStax^{TM} corn in the laboratory.   The F_2 leaf tissue bioassay showed that four of the 48 F_2 families from Rapides Parish and one of 29 families from Franklin Parish had a portion of larvae that survived after 7 days on leaf tissue of Genuity$^®$ VT Double Pro^{TM} with a total of ten and one survivors, respectively (Table 2.2).   Similarly, six families of the Rapides Parish population had a portion of larval that survived after 7 days on Genuity$^®$ SmartStax^{TM} leaf tissue with a total of 13 survivors, while none of the 29 families of the Franklin population survived on leaf tissue of Genuity$^®$ SmartStax^{TM}.   However, all live larvae of the 77 families on Genuity$^®$ VT Double Pro^{TM} were small ($\leq$ 2nd instars) and no large larvae ($\geq$ 3rd instars) survived for 7 days in the leaf tissue bioassay (Table 2.2).   A total of three large larvae from two families with an average of body mass of 5.7 mg were recovered on Genuity$^®$ SmartStax^{TM} leaf tissue.   Therefore, based on the criteria of Bt tolerant families described above, none of the 77 F_2 families in the Louisiana populations of fall armyworm qualified as potentially tolerant families.   The results of the F_2 leaf tissue bioassay suggested all of the 77 families were susceptible to the Genuity$^®$ VT Double Pro^{TM} and Genuity$^®$ SmartStax^{TM} hybrids.

2.3.3 Susceptibility of Florida Populations of Fall Armyworm to Genuity® VT Double Pro™ and Genuity® SmartStax™

A total of 72 F_2 two-parent families (or 144 feral individuals) of fall armyworm were established from Collier County, Florida during 2011 (Table 2.1). Among these families, 43 and 29 families were developed from the first and second field collections, respectively. All 72 families were assayed for susceptibility on leaf tissue of Genuity® VT Double Pro™ and Genuity® SmartStax™ in the laboratory. For the 43 families derived from the first collection, 20 families had a proportion of larvae that survived after 7 days on Genuity® VT Double Pro™ with a total of 93 survivors, and ten families had a proportion of larvae survived after 7 days on Genuity® SmartStax™ with a total of 50 survivors (Table 2.2). Among these survivors, a total of 30 large larvae with an average body mass of 9.0 mg/larva were recovered from nine families on Genuity® VT Double Pro™ and 21 large larvae with an average body mass of 8.9 mg/larva were found from four families on Genuity® SmartStax™ (Table 2.2). For the 29 families developed from the second collection, 11 families survived on Genuity® VT Double Pro™ with a total of 50 live larvae and 11 families survived on Genuity® SmartStax™ with a total of 47 survivors (Table 2.2). Among these survivors, 12 large larvae with an average body mass of 11.3 mg/larva were obtained from four families on Genuity® VT Double Pro™ and 17 large larvae with an average body mass of 10.8 mg/larva were observed from five families on Genuity® SmartStax™ (Table 2.2).

Based on the criteria for a tolerant family defined above, five out of the 72 Florida families were considered to be tolerating leaf tissue of Genuity® VT Double Pro™. Three of these families were from the first sampling including families 13, 30, and 32 and two families were from the second collection, i.e., families 17 and 22 (Table 2.3). For Genuity®

Table 2.2. Larval survival of two-parental family lines of Louisiana and Florida populations of *Spodoptera frugiperda* on leaf tissue of Genuity® VT Double PRO™ and Genuity® Smartstax™ corn.

Population	Location		No. F_2 lines assayed	No. lines survived	No. total survivors	No. lines with live large larvae	Total no. large larvae	Body mass of large larvae (mg/larva)
			Genuity® VT Double Pro™					
Louisiana	Rapides		48	4	10	0	0	—
	Franklin		29	1	1	0	0	—
	Subtotal		77	5	11	0	0	—
Florida	Collier	1st sampling	43	20	93	9	30	9.0
		2nd sampling	29	11	50	4	12	11.3
	Subtotal		72	31	143	13	42	9.6
Total			149	36	154	13	42	9.6
			Genuity® SmartStax™					
Louisiana	Rapides		48	6	13	2	3	5.7
	Franklin		29	0	0	0	0	—
	Subtotal		77	6	13	2	3	5.7
Florida	Collier	1st sampling	43	10	50	4	21	8.9
		2nd sampling	29	11	47	5	17	10.8
	Subtotal		72	21	97	9	38	9.7
Total			149	27	110	11	41	9.4

Note: Larvae that were ≥ 3rd instar were classified as large larvae.

SmartStax™, a total of four families were identified as potentially tolerant families which included three families (families 30, 32, and 45) from the first collection and one family (family 25) from the second sampling (Table 2.3). Attempts to establish laboratory colonies were made for all the potentially tolerant families. To establish laboratory colonies of the potentially tolerant families, survivors of each family after the leaf tissue bioassay were transferred onto meridic diet and reared individually until the pupal stage. Pupae of each family, if available, were then placed in paper containers for egg laying as described in Niu et al. (2013). Only two laboratory colonies of the nine families were successfully established from the survivors of the leaf tissue assays with F_2 generations. These two colonies were actually derived from the same F_2 family (family 32) of the first sampling, one for Genuity® VT Double Pro™ (thereafter labeled as FL1-32VT) and one for Genuity® SmartStax™ (FL1-32SS) (Table 2.4). In most cases, the very limited number of survivors and varied larval developmental and adult emergence times within a family resulted in the failure to establish laboratory colonies. The two colonies of fall armyworm that were considered to tolerate leaf tissue of Genuity® VT Double Pro™ or Genuity® SmartStax™ were re-evaluated twice for larval survival on leaf tissue of Genuity® VT Double Pro™ or Genuity® SmartStax™ leaf tissue. In the first re-evaluation, a total of 128 neonates of each colony were placed on the leaf tissue of their corresponding Bt corn products. After 7 days on Genuity® VT Double Pro™, only three small larvae ($\leq$ 2nd instar) of FL1-32VT were recovered (Table 4), while 32 small ($\leq$ 2nd instar) and two large ($\geq$ 3rd instar) of FL1-32SS were found from Genuity® SmartStax™ leaf tissue (Table 2.4). In the second leaf tissue re-evaluation, a total of 640 neonates were tested on Genuity® VT Double Pro™. After 7

38

days, 58 small larvae and two large larvae were obtained (Table 2.4).   Similarly, a total of

752 neonates were assayed against Genuity® SmartStax™ in the second re-evaluation and a

total of 100 survivors were recovered with 63 small and 27 large larvae after 7 days (Table

2.4).

Larvae of Bt-SS strain survived well on whole plants of the non-Bt corn hybrid with a

survivorship of $37.5 \pm 7.2\%$ and a leaf injury rating of 9 (Davis' 1-9 scale) after 15 days,

whereas no live larvae of Bt-SS were recovered from the two Bt corn hybrids with only little

leaf injury (a leaf injury rating of 1.19 ± 0.19) to Genuity® VT Double Pro™ and no damage

to Genuity® SmartStax™.   The results validated the high level of insecticidal activity of the

whole plants of the two Bt corn hybrids against fall armyworm in the greenhouse.   In the

tests with the two potentially 'tolerant' insect families, both greenhouse tests showed no

larvae of these two colonies could survive on the whole Bt plants of either Genuity® VT

Double Pro™ or Genuity® SmartStax™ and larvae of these two colonies caused no or little

leaf injury to the two Bt corn hybrids (Table 2.4).   In contrast, on non-Bt corn plants, the

family 32 caused an average of leaf injury rating of 5.42 ± 0.44 after 7 days.   After 13 days,

the leaf injury ratings increased to 7.67 ± 0.25 and an average of 2.3 ± 0.3 live larvae/ plant

were recovered from the non-Bt corn plants.   The results of the greenhouse tests showed that

the two potentially tolerant families were still susceptible to whole plants of the two

pyramided Bt corn products.   Greenhouse whole plant tests for the other seven potentially

tolerant families were not performed because of the failure to establish colonies of these

families.   Because of the similar performance of all of the nine potentially tolerant families

in the leaf tissue bioassay (Table 2.3), and because all larvae of FL1-32VT and FL1-32SS

Table 2.3. Family lines of *Spodoptera frugiperda* that were considered potentially tolerant to Genuity[®] VT Double PRO[TM] or Genuity[®] Smartstax[TM] corn products.

Location	Bt corn used in leaf tissue bioassay	Family line	No. of live larvae		Larval body mass (mg/larva)	
			Small	Large	Small	Large
	Genuity[®] VT Double Pro[TM]	13	3	4	1.7	9.5
Collier-FL1	Genuity[®] VT Double Pro[TM]	30	19	7	1.8	15.0
	Genuity[®] VT Double Pro[TM]	32	11	6	3.8	13.2
	Genuity[®] VT Double Pro[TM]	17	5	4	2.8	11.3
Collier-FL2	Genuity[®] VT Double Pro[TM]	22	15	5	1.6	13.6
	Genuity[®] SmartStax[TM]	30	3	4	1.3	8.3
Collier-FL1	Genuity[®] SmartStax[TM]	32	13	7	2.3	4.3
	Genuity[®] SmartStax[TM]	45	2	9	1.0	8.0
Collier-FL2	Genuity[®] SmartStax[TM]	25	3	7	3.7	12.0

Notes: 1) FL1 and FL2 were referred to the first sampling and second sampling respectively from Collier County in Florida.

2) Larvae that were $\leq 2^{nd}$ instar were referred as small larvae, while larvae that were $\geq 3^{rd}$ instar were classified as large larvae.

Table 2.4. Re-evaluations of susceptibility of two potentially tolerant families of *Spodoptera frugiperda* against Genuity® VT Double PRO™ and Genuity® Smartstax™ corn products.

Insect line	Bt maize	Leaf tissue re-evaluations						Whole plant tests					
		Trial 1			Trial 2			Trial 1			Trial 2		
		Total no. of larvae	No. of live larvae		Total no. of larvae	No. of live larvae		Total no. of larvae	No. of live larvae	Leaf injury ratings	Total no. of larvae	No. of live larvae	Leaf injury ratings
			Small	Large		Small	Large						
FL1-32VT	Genuity® VT Double Pro™	128	3	0	640	58	2	50	0	1.40±0.22	50	0	1.25±0.13
FL1-32SS	Genuity® SmartStax™	128	32	2	752	63	27	50	0	1.85±0.11	50	0	1.35±0.13

Notes: 1) FL1 and FL2 were referred to the first sampling and second sampling respectively from Collier County in Florida.

2) Larvae that were ≤ 2nd instar were referred as small larvae, while larvae that were ≥3rd instar were classified as large larvae.

were killed on whole plants of both Genuity® VT Double Pro™ and Genuity® SmartStax™ hybrids in both trials (Table 2.4), we proposed that the other seven potentially tolerant families to Bt corn leaf tissue are most likely also susceptible to the lethal action of whole plants of the two pyramided Bt corn hybrids.

2.4 Discussion

Baseline survival tests showed that the overall performance of fall armyworm was consistent and similar between the Bt-SS and the families derived from field collections. Larvae of the Bt-SS strain and the families derived from the field collections exhibited a relatively high mortality (~40%) after 7 days on non-Bt corn leaf tissue. However, they survived well on the meridic diet with an overall 7-day survivorship of 92.1%. In addition, larvae of the families that were tested in the greenhouse also survived well on whole plants of non-Bt corn after 13-15 days and caused heavy leaf damage. The results of the baseline bioassays indicated that the Bt-SS strain and the two-parent families established from the field collections were healthy. Natural mortality of insects reared on fresh plant materials or intact plants are common (Gassmann et al., 2011; Ghimire et al., 2011; Wangila et al., 2012). We believe that the cannibalistic behavior of fall armyworm larvae should not play a big role in the different mortality rates observed between the bioassays with meridic diet and fresh leaf tissue. Our preliminary bioassays showed that larval mortality of fall armyworm reared on non-Bt corn leaf tissue from one-to-four insects per assay well did not increase significantly (Huang et al., unpublished data). Such differences in larval mortality observed between the diet bioassay and the leaf tissue test could be due to the existence of some natural resistance factors in the plants to insects. Similar results were also reported in other

lepidopteran corn insect pests, such as European corn borer, southwestern corn borer, and

sugarcane borer, *Diatraea saccharalis* (F.) (Crambidae) (Huang et al., 2006).

Corn leaf tissues have been used in the F_2 screen for detecting Bt resistance alleles in

southwestern corn borer and sugarcane borer (Huang et al., 2007a; 2007b; 2012).   In those

studies, larval survival on corn leaf tissue in the F_2 screen was found to be highly correlated

with survival on whole Bt corn plants in the greenhouse.   However, in the current study we

found that both FL1-32VT and FL1-32SS had a high survivorship on Bt corn leaf tissue, but

could not survive on whole Bt plants in the greenhouse.   To confirm this observation, two

independent trials were conducted with both Bt corn products in the greenhouse and the

results were validated.   The difference in performance of fall armyworm on leaf tissue and

whole plants suggests that any survivors observed on leaf tissue in laboratory bioassays

should be re-examined carefully on the whole plants to confirm resistance.

A previous study had shown that both Genuity[®] VT Double Pro[TM] and Genuity[®]

SmartStax[TM] corn products were excellent against a Bt susceptible strain of fall armyworm

and almost 100% mortality was observed on both leaf tissue tests in the laboratory and whole

plant tests in the greenhouse (Niu et al., 2013).   However, in the current study, a

considerable number of larvae from many families survived in the leaf tissue bioassay,

especially of the populations collected from Florida.   All nine potentially tolerant families

identified in the leaf tissue in this study were derived from the Florida populations.   Such

survival on leaf tissue of the two pyramided Bt corn products may be due to a major

resistance allele to the Cry1F protein in Genuity[®] SmartStax[TM] and/or cross-resistance to

Cry1A.105 protein in Genuity[®] VT Double Pro[TM] and Genuity[®] SmartStax[TM].   In another

study, the same F_2 families of fall armyworm used in the current study were screened against a Cry1F corn hybrid (Huang et al., unpublished data). The F_2 screen on Cry1F corn leaf tissue showed that 67 out of these families possessed at least one major resistance allele to Cry1F corn plants, which included 21 families of the Louisiana populations and 46 families from the Florida populations. These families of fall armyworm were found to be highly resistant to both purified Cry1F protein and whole Cry1F plants. All of the nine families that showed a high larval survivorship and significant larval growth on leaf tissue of the pyramided Bt corn plants in the current study were among the families that were found to carry major resistance alleles to Cry1F corn plants (Huang et al., unpublished data). The results suggest that the Cry1F resistance alleles in these tolerant families could result in a significant growth and survival on the leaf tissue of the pyramided Bt plants. The Cry1A.105 in the pyramided Bt corn plants is a chimeric protein incorporating domains I and II from Cry1Ab and Cry1Ac and domain III from Cry1F (US-EPA, 2010). It is, therefore, possible that some level of cross-resistance could exist between Cry1F and Cry1A.105 because of the associations in their gene structures and the relative tolerance of fall armyworm to Cry1Ab/Cry1Ac proteins (US-EPA, 2001; Chilcutt et al., 2007; Hardke et al., 2011).

If Cry1F resistance and/or cross-resistance to Cry1A.105 in fall armyworm were present, it was not enough to allow larvae to survive on whole plants of Genuity® VT Double Pro™ or Genuity® SmartStax™, most likely because the activity of the Cry2Ab2 protein in the plants. Cry2Ab2 has a protein structures that differs from that of Cry1A.105 and these two proteins have different binding sites in the midguts of the target insects; thus Cry2Ab2 has a mode of

action distinct from that of Cry1F or Cry1A.105 (Storer et al., 2012b).   Several studies have

shown that usually a Cry1A resistant insect is not cross-resistant to Cry2Ab2 (Wu et al., 2009;

Sivasupramaniam et al., 2008; Brévault et al., 2009).   Similarly, a recent study also showed

that a highly Cry1F corn resistant population of fall armyworm collected from Puerto Rico

survived well on leaf tissue of Genuity® VT Double Pro™ and Genuity® SmartStax™ corn

hybrids in a 7-day-bioassay but could not survive on whole plants either of Genuity® VT

Double Pro™ or Genuity® SmartStax™ in the greenhouse (Niu et al., 2013; 2014).   The

results of these studies showed that these two pyramided Bt corn traits could provide some

value in managing the Cry1F resistance in fall armyworm.   However, once resistance occurs

to one Bt protein in a target insect, a pyramided Bt corn plant may just function as a

single-gene Bt trait and resistance management strategy in such situations should be

evaluated in future studies.   In summary, the results of the leaf tissue bioassays in the

laboratory and whole plant tests in the greenhouse showed that all 149 two-parent families of

fall armyworm collected from the three locations in Louisiana and Florida during 2011 were

susceptible to either Genuity® VT Double Pro™ or Genuity® SmartStax™ corn products.

The results suggest that the pyramided Bt corn products containing Genuity® VT Double

Pro™ and Genuity® SmartStax™ corn traits are effective against fall armyworm including

those possessing resistance alleles to Cry1F corn.

2.5 References

Adamczyk-Jr, J.J., Mahaffey, J.S., 2008. Efficacy of Vip3A and Cry1Ab transgenic traits in
    cotton against various lepidopteran pests. Fla. Entomol. 91, 570-575.

Brévault, T., Prudent, P., Vaissayre, M., Carrière., Y. 2009. Susceptibility of *Helicoverpa
    armigera* (Lepidoptera: Noctuidae) to Cry1Ac and Cry2Ab2 insecticidal proteins in four
    countries of the West African cotton belt. J. Econ. Entomol. 102, 2301-2309.

Buntiun, D., Flanders, K., 2012. 2012 Bt Corn Products for the Southeastern United States. Available at: http://www.caes.uga.edu/commodities/fieldcrops/gagrains/documents/2012BtcornSEBtc orntraitstableNov21.pdf.

Burkness, E.C., Dively, G., Patton, T., Morey, A.C., Hutchison, W. D., 2010. Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae) under field conditions. GM Crops 1, 1-7.

Davis, F.M., NG, S.S., Williams, W.P., 1992. Visual rating scales for screening whorl-stage corn for resistance to fall armyworm. Technical Bulletin 186, Mississippi Agric. Forestry Exp. Sta. 9 pp.

Chilcutt, C.F., Odvody, G.N., Correa, J.C., Remmers, J., 2007. Effects of *Bacillus thuringiensis* transgenic corn on corn earworm and fall armyworm (Lepidoptera: Noctuidae) densities. J. Econ. Entomol. 100, 327-334.

Gassmann, A.J., Petzold-Maxwell, J.L., Keweshan, R.S., Dunbar, M.W., 2011. Field-evolved resistance to Bt maize by western corn rootworm. PloS One 6, e22629.

Ghimire, M.N., Huang, F., Leonard, R.B., Head, G.P., Yang, Y., 2011. Susceptibility of Cry1Ab-susceptible and -resistant sugarcane borer to transgenic corn plants containing single or pyramided *Bacillus thuringiensis* genes. Crop Protect. 30, 74-81.

Hardke, J.T., Leonard, B.R., Huang, F., Jackson, R.E., 2011. Damage and survivorship of fall armyworm (Lepidoptera: Noctuidae) on transgenic field corn expressing *Bacillus thuringiensis* Cry proteins. Crop Protect. 30, 168-172.

Huang, F., Leonard, B.R., Gable, R.H., 2006. Comparative susceptibility of European corn borer, southwestern corn borer, and sugarcane borer (Lepidoptera: Crambidae) to Cry1Ab protein in a commercial Bacillus thuringiensis corn hybrid. J. Econ. Entomol. 99, 194-202.

Huang, F., Leonard, B.R., Andow, D.A., 2007a. F2 screen for resistance to a *Bacillus thuringiensis*-maize hybrid in the sugarcane borer (Lepidoptera: Crambidae). Bull. Entomol. Res. 97, 437-444.

Huang, F., Leonard, B.R., Cook, D.R., Lee, D.R., Andow, D.A., Baldwin, J.L., Tindall, K.V., Wu, X., 2007b. Frequency of alleles conferring resistance to *Bacillus thuringiensis* maize in Louisiana populations of southwestern corn borer (Lepidoptera: Crambidae). Entomol. Exp. Appl. 122, 53 - 58.

Huang, F., Andow, D.A., Buschman, L.L., 2011. Success of the high dose/refuge resistance management strategy after 15 years of Bt crops in North America. Entomol. Exp. Appl. 140, 1-16.

Huang, F., Ghimire, M.N., Leonard, B.R., Daves, C.D., Levy, R., Baldwin, J., 2012. Extended monitoring of resistance to *Bacillus thuringiensis* Cry1Ab maize in *Diatraea saccharalis* (Lepidoptera: Crambidae). GM Crop 3, 245-254.

James, C., 2013. Global status of commercialized biotech/GM crops: 2013. ISAAA Brief No. 45. ISAAA: Ithaca, NY, USA.

Matten, S.R., Head, G.P., Quemada, H.D., 2008. How government regulation can help or hinder the integration of Bt crops within IPM programs, pp. 27-39 In J. Romeis, A. M. Shelton and G. G. Kennedy [Eds.], Integration of Insect Resistant Genetically Modified Crops with IPM Programs. Springer Science Business Media B. V., New York, USA.

Monsanto, 2012. IRM grower guide: Insect resistance management for U.S. corn and cotton-growing areas. Available at: http://www.monsanto.com/products/Pages/insect-resistance-management.aspx.

Niu, Y., Meagher-JR, R.L., Yang, F., Huang, F., 2013. Susceptibility of field populations of the fall armyworm (Lepidoptera: Noctuidae) from Florida and Puerto Rico to purified Cry1F and corn leaf tissue containing single and pyramided Bt genes. Fla. Entomol. 96, 701-713.

Niu, Y., Yang, F., Dangal, V., Huang, F., 2014. Larval survival and plant injury of Cry1F-susceptible, -resistant, and -heterozygous fall armyworm (Lepidoptera: Noctuidae) on non-Bt and Bt corn containing single or pyramided genes. Crop Protect. 59, 22-28.

Pashley, D.P., 1986. Host associated genetic differentiation in fall armyworm (Lepidoptera: Noctuidae): a sibling species complex. Ann. Entomol. Soc. Amer. 79, 898-904.

Pashley, D.P., 1988. Quantitative genetics, development, and physiological adaptation in host strains of fall armyworm. Evolution 42, 93-102.

Pashley, D.P., Johnson, S.J., Sparks, A.N., 1985. Genetic population structure of migratory moths: the fall armyworm (Lepidoptera: Noctuidae). Ann. Entomol. Soc. Amer. 78, 756-762.

SAS Institute Inc., 2010. SAS/STAT: 9.3 User's Third Edition SAS Institute Inc., Cary, NC.

Sivasupramaniam, S., Moar, W.J., Ruschke, L.G., Osborn, J.A., Jiang, C., Sebaugh, J.L., Brown, G.R., Shappley, Z.W., Oppenhuizen, M.E., Mullins, J.W., Greenplate, J.T., 2008. Toxicity and characterization of cotton expressing *Bacillus thuringiensis* Cry1Ac and Cry2Ab2 proteins for control of lepidopteran pests. J. Econ. Entomol. 101, 546-54.

Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W., Huckaba, R.M., 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 103, 1031-1038.

Storer, N.P., Kubiszak, M.E., King, J.E., Thompson, G.D., Santos, A.C., 2012a. Status of resistance to Bt maize in *Spodoptera frugiperda*: Lessons from Puerto Rico. J. Invert. Pathol. 110, 294-300.

Storer, N.P., Thompson, G.D., Head, G.P., 2012b. Application of pyramided traits against Lepidoptera in insect resistance management for Bt crops. GM Crops 3, 154-162.

Syngenta, 2012. Corn trait Information: Agrisure Viptera™ 3111. Available at: http://www.syngenta.com/country/us/en/Seeds/Traits/CornTraits/Pages/content_authoring_Agrisurevip3111.aspx.

US-EPA (United States Environmental Protection Agency), 2001. Biopesticide registration action document: *Bacillus thuringiensis* plant-incorporated protectants. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm.

US-EPA (United States Environmental Protection Agency), 2005. Biopesticide registration action document: *Bacillus thuringiensis* Cry1F Corn. Available at: http://www.epa.gov/opp00001/biopesticides/ingredients/tech_docs/brad_006481.pdf.

US-EPA (United States Environmental Protection Agency), 2010. Biopesticide registration action document: *Bacillus thuringiensis* Cry1A.105 and Cry2Ab2 insecticidal proteins and the genetic material necessary for their production in corn. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/mon-89034-brad.pdf.

Wangila, D.S., Leonard, B.R., Bai, Y., Head, G.P., Huang, F. 2012. Larval survival and plant injury of Cry1Ab-susceptible, -resistant, and -heterozygous genotypes of the sugarcane borer on transgenic corn containing single or pyramided Bt genes. Crop Protect. 42, 108-115.

Wu, X., Huang, F., Leonard, B.R., Moore, S.H. 2007. Evaluation of transgenic *Bacillus thuringiensis* corn hybrids against Cry1Ab-susceptible and -resistant sugarcane borer (Lepidoptera: Crambidae). J. Econ. Entomol. 100, 1880-1886

Wu, X., Leonard, B.R., Zhu, Y. C., Abel, C.A., Head, G.P., Huang, F. 2009. Susceptibility of Cry1Ab-resistant and -susceptible sugarcane borer (Lepidoptera: Crambidae) to four *Bacillus thuringiensis* toxins. J. Invertbr. Pathol. 100, 29-34.

CHAPTER 3. SUSCEPTIBILITY OF LOUISIANA AND FLORIDA POPULATIONS OF *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTUIDAE) TO TRANSGENIC AGRISURE® VIPTERA™ 3111 CORN[2]

3.1 Introduction

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is indigenous to the subtropical and tropical regions of the Western Hemisphere from the United States to Argentina (Pashley et al., 1985; Pashley, 1986; 1988).   It is distributed throughout most of the United States east of the Rocky Mountains, but it is believed to be able to overwinter successfully only in southern Florida and southern Texas (Sparks, 1979; Buntin, 1986).   Every year, populations from overwintered areas migrate into various regions across the country (Belay et al., 2012).   This notorious pest has a wide range of > 80 host plants, including many major field crops such as maize, cotton, soybeans, sorghum, and rice (Sparks, 1979; Knipling, 1980; Rojas et al., 2004; Wyckhuys and O'Neil, 2006).   Larvae of this pest can cause great damage by consuming foliage and also can burrow into the growing point (bud, whorl, etc.), destroying the growth potential of plants, or clipping the leaves. Management of fall armyworm in field maize with conventional chemical insecticides is inconsistent (Young, 1979; Guillebeau and All, 1990) and resistance of the insect to commonly used insecticides has been reported in many areas in the United States (Adamczyk-Jr et al., 1999).

Since 1996, transgenic maize hybrids expressing *Bacillus thuringiensis* (Bt) endotoxins have been grown extensively in the United States and many other countries in the world (James, 2013; Huang et al., 2011b).   In 2012, 67% (or 64.5 million acres) of the U.S. field

---

[2] Reprinted with permission by the Crop Protection

corn was planted to Bt maize carrying one or more Bt proteins (USDA-NASS, 2012).

Before 2010, only single-gene Bt maize for a target species was commercially planted.

These single-gene Bt maize hybrids are very effective for controlling stalk borers such as the

European corn borer, *Ostrinia nubilalis* (Hübner), and southwestern corn borer, *Diatraea*

grandiosella Dyar, but most are just partially effective against fall armyworm.   Studies have

shown that all single-gene Bt maize products do not produce a "high dose" for fall armyworm

(Adamczyk-Jr and Mahaffey, 2008; Chilcutt et al., 2007; Hardke et al., 2011; Huang et al.,

2011a; US-EPA, 2001).   For this reason, fall armyworm is not listed as a target for

single-gene Bt maize products except for Herculex®I (US-EPA, 2001; 2005).   Unfortunately,

but not unexpected, with the wide use of Herculex®I maize in Puerto Rico, field resistance of

fall armyworm to Cry1F maize occurred in 2006 in the area (Storer et al., 2010).

To broaden the target spectrum and delay resistance evolution, a gene-pyramiding

strategy has been used to develop transgenic plants containing two or more Bt proteins with

dissimilar modes of actions but effective against the same target pest (Ghimire et al., 2011;

Monsanto, 2012; Eggerling and Jackson, 2012).   Such Bt maize hybrids expressing

pyramided Bt genes (e.g. Agrisure® Viptera™ 3111) have been commercially grown for

controlling both above- and below-ground maize insect pests in the United States since 2010

(US-EPA, 2009; 2010).   Compared to single-gene Bt maize, pyramided Bt maize hybrids are

expected to be more effective for controlling some Noctuidae species such as the corn

earworm, *Helicoverpa zea* (Boddie) and fall armyworm.   Both corn earworm and fall

armyworm are listed as target species in all currently commercialized pyramided Bt maize

products in the United States (US-EPA, 2009; 2010; Monsanto, 2012; Syngenta, 2012).   The

main merit of pyramided Bt crop technology is the relatively low survival of heterozygous resistant-insect individuals due to the high efficiency of multiple Bt proteins (Ives et al., 2011). Because pyramided Bt crops have two or more Bt proteins against a target pest, and thus the probability that an insect possesses alleles that resist to all Bt proteins in the pyramided plants should be extremely rare. Although evolution of resistance to plants with pyramided Bt genes is expected to be much slower relatively to single-gene Bt plants, nonetheless it is driven by the same evolutionary process as against single-gene Bt crops (Ives et al., 2011).

During 2011, a total of 150 F_2 two-parent family lines of fall armyworm were developed by using single-pair matings of field individuals collected from Louisiana and Florida. Among these lines, 142 lines were examined for resistance to Cry1F maize plants using an F_2 screen method. The F_2 screen showed that 67 out of the 142 family lines possessed at least one major resistance allele to a commercial Cry1F maize hybrid (Huang et al., unpublished data). Compared to a laboratory susceptible strain, the resistant lines have demonstrated highly resistant to both purified Cry1F protein and Cry1F maize plants. Therefore, it was interesting to know the performance of these family lines of fall armyworm on the second generation pyramided Bt maize products. The objective of this study was to determine the susceptibility of these field derived two-parent family lines of fall armyworm to a pyramided Bt maize hybrid containing Agrisure[®] Viptera[TM] 3111 traits.

3.2 Materials and Methods

3.2.1 Insect Collection and Rearing

Third to fifth instar larvae of fall armyworm were collected from sorghum fields in Franklin (32°08' N; 91°41'W) and Rapides (31°10'35.99"N; 92°23'24.24" W) parishes in Louisiana during September 14-22, 2011 and from non-Bt sweet corn fields in Collier County in Florida (26°28'N, 81°26'W) during October 16-27, 2011.   In each location, larvae were sampled in two different times with a one- to two-week interval between the two samplings in each location.   All field-collected larvae were reared individually on a meridic diet (WARD'S Stonefly Heliothis diet, Rochester, NY) in 30-ml plastic cups (Fill-Rite, Newark, NJ) until pupal stage using the method as described in Niu et al. (2013).

3.2.2 Single-Pairing and Establishment of Two-Parent Families

The procedures used to establish two-parent families of fall armyworm were similar as used for sugarcane borer, *Diatraea saccharalis* (F.) (Huang et al., 2007).   Pupae developed from field-collected larvae were sexed into males and females.   Newly emerged virgin male and female adults were single-paired in 2- or 3.8-L paper containers (Huhtamaki Foodservice, De Soto, KS) for adult mating and oviposition.   The containers were maintained in an environmental chamber at 28^0C, >90% RH and a 14:10 h (L: D) photoperiod.   Progeny (F_1) produced from each single-pair was considered as a two-parent family.   These F_1 neonates of each two-parent family were reared individually in the 30-ml cups containing the meridic diet as mentioned above. F_1 adults within each single family were then sib-mated in 3.8L cardboard cartons (Neptune Paper Products, Newark, NJ) to produce F_2 offspring for each family.   The number of viable F_1 pupae of each two-parent family to produce F_2 progeny

ranged from 55 to 80 with an average of 76.5 ± 1.0 (mean ± SE) for the Louisiana

populations and 50 to 80 with an average of 67.9 ± 1.7 for the Florida populations.

3.2.3 Source of Bt and Non-Bt Corn Materials

Leaf tissue of a corn hybrid, NK N78N-3111 (Syngenta, Minnetonka, MN), containing

Agrisure[®] Viptera[TM]3111 traits was used in a laboratory bioassay.   Agrisure[®] Viptera[TM]

3111corn plants contain Vip3A and Cry1Ab proteins for controlling above-ground

lepidopteran species including fall armyworm and mCry3A protein for managing

below-ground rootworms, *Diabrotica* spp (Coleoptera: Chrysomelidae).   Vip3A is an

exotoxin generated during the vegetative stage of the Bt bacteria, whereas Cry proteins (e.g.

Cry1Ab) are produced during sporulation (Yu et al., 1997; Kurtz, 2010).   Vip3A shows no

sequence homology with any known Cry proteins, indicating no cross-resistance between

Vip3A and Cry1Ab (Lee et al., 2003; Burkness et al., 2010).   In addition, a genetically

closely related non-Bt corn hybrid, Agrisure[®] NK N78N-GT (Minnetonka, MN), was used to

establish the baseline survival of fall armyworm.   Both Bt and non-Bt corn hybrids were

planted in 18.9L pots each containing approximately 5kg of a standard potting soil mixture in

a greenhouse located at the Louisiana State University Agricultural Center in Baton Rouge,

Louisiana, USA as described in Wu et al. (2007).   Expression of Vip3A and Cry1Ab

proteins in Agrisure[®] Viptera[TM]3111 plants was confirmed using ELISA-based assays

(EnviroLogix, Quantiplate[TM] kits, Portland, ME).

3.2.4 Leaf Tissue Bioassay

Larval survival of each two-parent family of fall armyworm was assayed in 32-well

trays (Bio-Smart-32, C-D International, Pitman, NJ) containing corn leaf tissue dissected

from leaves of V4-V10 stages of greenhouse grown corn plants of Agrisure[®] Viptera[TM]3111 plants.   Based on our preliminary study involved in consideration of both larval cannibalism effect and labor intensity needed in the laboratory bioassays, four individuals of fall armyworm in each well were considered to be an appropriate number adopted in the leaf tissue bioassay to minimize larval cannibalism (Niu et al. 2013).   In the bioassay, a total of 96 F_2 neonates of each two-parent family were examined in 24 wells (four neonates/ well) each containing 3-4 pieces of approximately 3-cm fresh leaf tissue.   Bioassay trays containing leaf tissue and insects were then placed in environmental chambers maintained at 28°C, ~50% RH, and a 16:8 h (L: D) photoperiod.   Leaf tissue was replaced every 3 days. Larval survival was checked at 7 days after inoculation.

In addition, baseline survival of a Bt-susceptible strain (Bt-SS) of fall armyworm on leaf tissue of the non-Bt and Bt corn hybrids were also determined using the same method as described above.   The Bt-SS strain was established from larvae collected from non-Bt corn fields in Hendry County, Florida, USA during 2011 (Niu et al., 2013).   It has been documented to be susceptible to most Bt corn traits including Herculex[®]I; Genuity[®]VT Double Pro[TM], VT Triple Pro[TM], and SmartStax[TM]; as well as Agrisure[®] Viptera[TM] 3111 (Niu et al., 2013).   Moreover, larval survival of seven two-parent families developed from the field collections was also assayed in leaf tissue of four non-Bt corn hybrids including Agrisure[®] NK N78N-GT, Pioneer 31G66 (Pioneer Hi-Bred, Johnston, IA), DKC 67-86 (Monsanto, St. Louis, MO), and DKC 61-22(Monsanto, St. Louis, MO).   For each baseline bioassay, there were four replications with 32 larvae in each replication and larval survival was checked after 7 days (Niu et al. 2013).   Baseline survival data were transformed with

arcsine $(x)^{0.5}$, followed by a one-way analysis of variance.   Larval survival of the insect

sources (Bt-SS, Louisiana, and Florida) on non-Bt corn leaf tissue was separated with LSD

tests at α= 0.05 level (SAS Institute, 2010).   Furthermore, to verify the viability of the

two-parent families derived from the field collections, larval survival of the seven F_2 families

together with the Bt-SS strain of fall armyworm was also determined on a meridic diet as

described in Niu et al. (2013).

3.3 Results

3.3.1 Baseline Survivorship

Larvae of all three sources of fall armyworm survived well on the meridic diet with a

7-day survivorship rate of 89.1 ± 1.7% for the Bt-SS strain, 90.3± 1.4% for the family lines

sampled from Florida, and 96.8± 1.3% from Louisiana, suggested that the family lines

developed from the single-pairings of field-collected individuals were viable.   On leaf tissue,

larval survivorship rate of the susceptible population of fall armyworm on non-Bt maize was

58.6 ± 9.7% after 7 days, compared to zero on Agrisure[®] Viptera[TM] 3111 maize, suggesting

that Agrisure[®] Viptera[TM] 3111 maize leaf tissue expressed a sufficient level of Bt proteins to

kill susceptible fall armyworm and thus could be used as a "discriminating dose" to identify

family lines that were possibly resistant/tolerant to Agrisure[®] Viptera[TM] 3111.   Larval

survival on non-Bt maize leaf tissue was not significantly affected by insect sources ($F = 0.28$;

df = 2, 53; $P = 0.7594$).   Survivorship rate of larvae after feeding 7 days on non-Bt leaf

tissue was 59.2±1.8% for the family lines collected from Louisiana and 61.4 ± 2.6% for the

lines sampled from Florida, which was not significantly ($P > 0.05$) different compared to that

(58.6 ± 9.7%) observed for the Bt-SS strain.

3.3.2 Susceptibility of Louisiana Populations of Fall Armyworm to Agrisure® Viptera™ 3111

A total of 78 F_2 two-parental family lines (156 feral individuals) of fall armyworm were established from single-pairings of moths derived from feral larvae collected from two locations in Louisiana in 2011 (Table 3.1). Among these lines, 49 and 29 were established from single-pairings of insects collected from Rapides and Franklin parishes, respectively. All of these F_2 family lines were examined for susceptibility to Agrisure® Viptera™ 3111 maize leaf tissue in the laboratory. The laboratory bioassays showed that all 7,488 neonates from the 78 family lines (96 neonates/line $\times$ 78 = 7,488) were killed within 7 days on Agrisure® Viptera™ 3111 maize leaf tissue, suggesting all of these family lines were susceptible to the Bt plants.

Table 3.1. Larval survival of F_2 two-parental family lines of Louisiana and Florida populations of *Spodoptera frugiperda* on leaf tissue of Agrisure® Viptera™ 3111 Bt maize

Population	Location	Number of F_2 lines assayed	Total number of neonates assayed	7-day survival	
				Number of lines surviving	Number of live insects
Louisiana	Rapides	49	4,704	0	0
	Franklin	29	2,784	0	0
	Subtotal	78	7,488	0	0
Florida	Collier	72	6,912	0	0
Total		150	14,400	0	0

3.3.3 Susceptibility of Florida Populations of Fall Armyworm to Agrisure® Viptera™ 3111

A total of 72 F_2 two-parent family lines (144 feral individuals) of fall armyworm were established with single-pairings of insects from two field collections in Collier County, Florida during 2011 (Table 3.1). Larval survival of these F_2 lines was assayed on Agrisure®

Viptera^{TM} 3111 maize leaf tissue in the laboratory.   As observed in the Louisiana populations, the leaf tissue bioassays also showed that none of the 6,912 F$_2$ neonates from the 72 family lines of the Florida populations survived for 7 days, indicating that all of the 72 family lines sampled from Florida were also susceptible to Agrisure$^®$ Viptera^{TM} 3111.

3.4 Discussion

Cannibalism is a prevalent behavior of fall armyworm larvae, especially for late instars. Several studies have shown that behavior of cannibalism is frequent among fall armyworm larvae in laboratory culture and in the field (Chapman et al., 1999a; 1999b; 2000). Compared to other traditional bioassays (e.g. dose-response bioassay, discriminating dose bioassay), cost, especially labor cost, is a major limitation of the bioassays using single-pairing families (Andow et al., 2000; Huang, 2006).   Based on a preliminary study, effect of larval cannibalism of fall armyworm on 7-day larval survivorship was not significant when two to four individuals were reared together on the leaf tissue in the same types of bioassay wells as used in the current study (Huang et al., unpublished data). Therefore consideration of the effect of larval cannibalism and labor cost, in the current study, four neonates of fall armyworm were collectively assayed in a well.   The labor intensity of the bioassay procedures used in this study was manageable with two to four labors in our laboratory.

In the current study, all 14,400 larvae of the 150 two-parental family lines developed from single-pairings of fall armyworm collected from Louisiana and Florida were killed after 7 days on leaf tissue of Agrisure$^®$ Viptera^{TM}3111 Bt maize, suggesting the field populations of fall armyworm from both states were highly susceptible to Agrisure$^®$ Viptera^{TM}3111 Bt maize.

In a separate study, the same bioassay procedures were used to detect major resistance alleles to Cry1F Bt maize in the same F_2 two-parental family lines (Huang et al., unpublished data). The results of that study showed that 67 out of these family lines possessed at least one major resistance allele to a commercial Cry1F maize hybrid. The resistant family lines including 21 lines collected from Louisiana and 46 lines from Florida survived well on whole plants of Cry1F maize plants and were also highly resistant to purified Cry1F protein. The results of the current study demonstrated that the pyramided Bt maize hybrid containing Agrisure® Viptera™3111 traits is effective against fall armyworm including those possessing Cry1F resistance alleles and thus should provide a means for managing Cry1F resistance in the insect.

The 'high dose/refuge' strategy aimed at elongating the efficacy of Bt crops is the primary method currently used for Bt resistance management in the United States. This strategy requires that Bt plants express a sufficiently high concentration of Bt proteins to kill heterozygous resistant individuals of target pest species (Andow and Hutchison, 1998; US-EPA, 2001). The most direct way to validate the 'high dose' assumption is to examine the survival of resistant heterozygotes of the target pest on Bt maize plants (Wu et al., 2007). However, resistant strains for many target insects are not available, especially for resistance to pyramided traits. Actually we still do not have a clear definition of "high dose" for pyramided Bt traits. Therefore, indirect criteria of 'high dose' are proposed to evaluate the high dose status of Bt crops (US-EPA, 2001). Most commercial Bt maize products are believed to produce "high dose" for the two most important maize stalk borers in the United States, European corn borer and southwestern corn borer (Huang et al., 2011a). Recently,

Burkness et al. (2010) reported that pyramided Bt sweet corn containing Vip3A and Cry1Ab genes was also very effective against corn earworm in five-year field trials.   Based on these field tests, the pyramided maize expressing both Vip3A and Cry1Ab was presumed to produce a "high dose" for corn earworm (Burkness et al., 2010).   Thus far, the high dose qualification for fall armyworm has not been documented in any Bt maize products.   In the current study, survival of 14,400 neonates from 150 two-parent family lines of fall armyworm was evaluated on Agrisure$^®$ Viptera^{TM} 3111 plants in the F_2 screen.   Actually, during the laboratory bioassays we observed that all (14,400) expect one F_2 larvae from the 150 family lines were killed within only three days.   The one that survived at 3rd day was very weak and dead on 7th day.   In addition, a recent study also showed that a highly Cry1F-maize resistant population of fall armyworm collected from Puerto Rico was susceptible to Agrisure$^®$ Viptera^{TM} 3111 maize in both leaf tissue bioassays in the laboratory and whole plant tests in the greenhouse (Niu et al., 2013).   Larvae of all three genotypes, Cry1F-susceptible, -resistant, and -heterozygous, of fall armyworm couldn't survive on both leaf tissue and whole plants of Agrisure$^®$ Viptera^{TM} 3111maize.   Although both the current and the previous studies were not designed to evaluate the high dose assumption, the results of these studies suggest that Agrisure$^®$ Viptera^{TM} 3111 maize is very effective and likely expresses a "high-dose" for fall armyworm.   The qualification of "high dose" would make Agrisure$^®$ Viptera^{TM} 3111 maize traits very valuable in management of fall armyworm, a pest that is often found to be tolerant to most other Bt maize products.

3.5 References

Adamczyk-Jr, J.J., Leonard, B.R., Graves, J.B., 1999. Toxicity of selected insecticides to fall armyworm (Lepidoptera: Noctuidae) in laboratory bioassay studies. Fla. Entomol. 82, 230-236.

Adamczyk-Jr, J.J., Mahaffey, J.S., 2008. Efficacy of Vip3A and Cry1Ab transgenic traits in cotton against various lepidopteran pests. Fla. Entomol. 91, 570-575.

Andow, D.A., Hutchison, W.D., 1998. Bt-corn resistance management. Now or never: Serious new plans to save a natural pest control (ed. by Union of Concerned Scientists), pp. 19-66. Cambridge, MA, USA.

Andow, D.A., Olson, D.M., Hellmich, R.L., Alstad, D.N., Hutchison, W.D., 2000. Frequency of resistance to *Bacillus thuringiensis* toxin Cry1Ab in an Iowa population of European corn borer (Lepidoptera: Crambidae). J. Econ. Entomol. 93, 26-30.

Belay, D.K., Clark, P.L., Skoda, S.R., Isenhour, D.J., Ochoa, J.M., Gianni, C., Foster, J.E., 2012. Spatial genetic variation among *Spodoptera frugiperda* (Lepidoptera: Noctuidae) sampled from the United States, Puerto Rico, Panama, and Argentina. Ann. Entomol. Soc. Amer. 105, 359-367.

Buntin, G.D., 1986. A review of plant response to fall armyworm, *Spodoptera frugiperda* (J. E. Smith), injury in selected field and forage crops. Fla. Entomol. 69, 549-559.

Burkness, E.C., Dively, G., Patton, T., Morey, A.C., Hutchison, W.D., 2010. Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae) under field conditions. GM Crops, 1, 1-7.

Chapman, J.W., Williams, T., Escribano, A., Caballero, P., Cave, R.D., Goulson, D., 1999a. Age-related cannibalism and horizontal transmission of a nuclear polyhedrosis virus in larval *Spodoptera frugiperda*. J. Ecol. Entomol. 24, 268-275.

Chapman, J.W., Williams, T., Escribano, A., Caballero, P., Cave, R.D., Goulson, D., 1999b. Fitness consequences of cannibalism in the fall armyworm, *Spodoptera frugiperda*. Behav. Ecol. 10, 298-303.

Chapman, J.W., Williams, T., Martinez, A.M., Cisneros, J., Caballero, P., Cave, R.D., Goulson, D., 2000. Does cannibalism in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) reduce the risk of predation? Behav. Ecol. Sociobiol. 48, 321-327.

Chilcutt, C.F., Odvody, G.N., Correa, J.C., Remmers, J., 2007. Effects of *Bacillus thuringiensis* transgenic corn on corn earworm and fall armyworm (Lepidoptera: Noctuidae) densities. J. Econ. Entomol. 100, 327-334.

Eggerling, K., Jackson, M., 2012. Select seed, 2012 product guide. Available at: http://www.selectseed.com/media/2012guide/2012SelectSeedFinalProductGuide.pdf.

Ghimire, M.N., Huang, F., Leonard, R.B., Head, G. P., Yang, Y., 2011. Susceptibility of Cry1Ab-susceptible and -resistant sugarcane borer to transgenic corn plants containing single or pyramided *Bacillus thuringiensis* genes. Crop Protect. 30, 74-81.

Guillebeau, L.P., All, J.N., 1990. Use of pyrethroids, methomyl, and chlorpyrifos to control fall armyworm (Lepidoptera: Noctuidae) in whorl stage field corn, sweet corn, and sorghum. Fla. Entomol. 74, 261-270.

Hardke, J.T., Leonard, B.R., Huang, F., Jackson, R.E., 2011. Damage and survivorship of fall armyworm (Lepidoptera: Noctuidae) on transgenic field corn expressing *Bacillus thuringiensis* Cry proteins. Crop Protect. 30, 168-172.

Huang, F., 2006. Detection and monitoring of insect resistance to transgenic Bt crops. Ins. Sci. 13, 73-84.

Huang, F., Andow, D.A., Buschman, L.L., 2011a. Success of the high dose/refuge resistance management strategy after 15 years of Bt crops in North America. Entomol. Exp. App. 140, 1-16.

Huang, F., Ghimire, M.N., Leonard, .BR., Wang, J., Daves, C., Levy, R., Cook, D., Head, G.P., Yang, Y., Temple, J., Ferguson, R., 2011b. F_2 screening for resistance to pyramided *Bacillus thuringiensis* maize in Louisiana and Mississippi populations of *Diatraea saccharalis* (Lepidotera: Crambidae). Pest Manag. Sci. 67, 1269-1276.

Huang, F., Leonard, B.R., Andow, D.A., 2007. F_2 screen for resistance to a *Bacilllus thuringiensis*-maize hybrid in the sugarcane borer (Lepidoptera: Crambidae). Bull. Entomol. Res. 97, 437-444.

Ives, A.R., Glaum, P.R., Ziebarth, N.L., Andow, D.A., 2011. The evolution of resistance to two-toxin pyramided transgenic crops. Ecol. App. 21, 503-515.

James, C., 2013. Global status of commercialized biotech/GM crops: 2013. ISAAA Brief No. 45. ISAAA: Ithaca, NY, USA.

Knipling, E.F., 1980. Regional management of the fall armyworm a realistic approach. Fla. Entomol. 63, 468-480.

Kurtz, R.W., 2010. A review of Vip3A mode of action and effects on Bt cry protein-resistant colonies of Lepidopteran larvae. Southwest. Entomol. 35, 391-394.

Lee, M.K., Walters, F.S., Hart, H., Palekar, N., Chen, J., 2003. The model of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab δ-endotoxin. App. Environ. Microbiol. 69, 4648-4657.

Monsanto, 2012. IRM grower guide: insect resistance management for U.S. corn and cotton-growing areas. Available at: http://www.monsanto.com/products/Pages/insect-resistance-management.aspx.

Niu, Y., Meagher-Jr, R., Yang, F., Huang, F., 2013. Susceptibility of field populations of the fall armyworm (Lepidoptera: Noctuidae) from Florida and Puerto Rico to purified Cry1F protein and corn leaf tissue containing single and pyramided Bt genes. Fla. Entomol. 96, 701-713.

Pashley, D.P., 1986. Host associated genetic differentiation in fall armyworm (Lepidoptera: Noctuidae): a sibling species complex. Ann. Entomol. Soc. Amer. 79, 898-904.

Pashley, D.P., 1988. Quantitative genetics, development, and physiological adaptation in host strains of fall armyworm. Evolution 42, 93-102.

Pashley, D.P., Johnson, S.J., Sparks, A.N., 1985. Genetic population structure of migratory moths: the fall armyworm (Lepidoptera: Noctuidae). Ann. Entomol. Soc. Amer. 78, 756-762.

Rojas, J.C., Virgen, A., Malo, E.A., 2004. Seasonal and nocturnal flight activity of *Spodoptera frugiperda* males (Lepidoptera: Noctuidae) monitored by pheromone traps in the coast of Chiapas, Mexico. Fla. Entomol. 87, 496-503.

SAS Institute Inc, 2010. SAS/STAT: 9.3 User's Third Edition SAS Institute Inc, Cary, NC.

Sparks, A.N., 1979. A review of the biology of the fall armyworm. Florida Entomol. 62**,** 82-87.

Steiner, H.Y., 2006. Petition for the determination of non-regulated status corn rootworm protected transformation event MIR604. Available at: http://www.aphis.usda.gov/brs/aphisdocs/04_36201p.pdf.

Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W., Huckaba, R.M., 2010. Discovery and characterization of field resistance to Bt maize: Spodoptera frugiperda (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 103, 1031-1038.

Syngenta, 2012. Corn trait Information: Agrisure Viptera™ 3111. Available at: http://www.syngenta.com/country/us/en/Seeds/Traits/CornTraits/Pages/content_authoring_Agrisurevip3111.aspx.

USDA-NASS, 2012. (National Agricultural Statistics Service). Acreage. USDA, Washington DC. USA . Available at: http://usda01.library.cornell.edu/usda/current/Acre/Acre-06-29-2012.pdf.

US-EPA (United States Environmental Protection Agency), 2001. Biopesticide registration action document: *Bacillus thuringiensis* plant-incorporated protectants. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm.

US-EPA (United States Environmental Protection Agency), 2005. Biopesticide registration action document: *Bacillus thuringiensis* Cry1F Corn. Available at: http://www.epa.gov/opp00001/biopesticides/ingredients/tech_docs/brad_006481.pdf.

US-EPA (United States Environmental Protection Agency), 2009. Biopesticide registration action document: *Bacillus thuringiensis* Vip3Aa20 insecticidal protein and the genetic material necessary for its production (*via* elements of vector pNOV1300) in event MIR162 maize (OECD unique identifier: SYN-IR162-4). Available at: http://www.epa.gov/opp00001/biopesticides/ingredients/tech_docs/brad_006599.pdf.

US-EPA (United States Environmental Protection Agency), 2010. Biopesticide registration action document: *Bacillus thuringiensis* Cry1A.105 and Cry2Ab2 insecticidal proteins and the genetic material necessary for their production in corn. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/mon-89034-brad.pdf.

Wu, X., Huang, F., Leonard, B.R., Moore, S.H., 2007. Evaluation of transgenic *Bacillus thuringiensis* corn hybrids against Cry1Ab-susceptible and -resistant sugarcane borer (Lepidoptera: Crambidae). J. Econ. Entomol. 100, 1880-1886.

Wyckhuys, K.A.G., O'Neil, R.J., 2006. Populations dynamics of *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) and associated arthropod natural enemies in Honduran subsistence maize. Crop Protect. 25, 1180-1190.

Young, J.R., 1979. Fall armyworm: control with insecticides. Fla. Entomol. 62, 130-133.

Yu, C., Mullins, M.A., Warren, G.W., Koziel, G.M., Estruch, J.J., 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. App. Environ. Microbiol. 63, 532-553.

CHAPTER 4. OCCURRENCE, DISTRIBUTION, AND EAR DAMAGE OF *HELICOVERPA ZEA* (LEPIDOPTERA: NOCTUIDAE) IN MIXED PLANTINGS OF NON-BT AND BT CORN CONTAINING GENUITY® SMARTSTAX™ TRAITS[3]

4.1 Introduction

Pyramided Bt corn (*Zea mays L.*) products containing multiple *Bacillus thuringiensis* (Bt) proteins targeting the same pest species have been commercially planted in the United States since 2010 (US-EPA, 2010; Matten et al., 2012; Wangila et al., 2013). Relative to single Bt gene corn products, these pyramided Bt corn hybrids are expected to perform better in protecting crops against target pests and delaying resistance evolution (Roush, 1998; Zhao et al., 2003; Ives et al., 2011; Yang et al., 2013). The multiple Bt proteins in these pyramided Bt products exhibit different modes of toxicity (Monsanto, 2012; Syngenta, 2012). Therefore, the likelihood of resistance evolution to a pyramided product is expected to be lower than for single trait products (Roush, 1998; Zhao et al., 2003; Monsanto, 2012). One of the most popular pyramided Bt corn traits in the commercial market is Genuity® SmartStax™ which expresses six Bt proteins: Cry1A.105/Cry2Ab2 (MON 89034), Cry1F (TC1507), Cry3Bb1 (MON 88017), Cry34/35Ab1 (DAS-59122) (Monsanto, 2012).

Although additional studies are still needed to document if the pyramided Bt corn products qualify as 'high dose' as defined by the U.S. EPA FIFRA Scientific Advisory Panel (FIFRA, 1998; US-EPA, 2001), a few field studies have shown that the pyramided Bt corn products are usually more effective for the target pests, especially for the noctuid species, such as corn earworm, *Helicoverpa zea* (Boddie) and fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Burkness et al., 2010, Yang et al. 2013). Based on data from insect movement

---

[3] Reprinted with permission by the Crop Protection

and survival of corn borers on pyramided Bt corn as well as the grower's compliance issues

associated with the use of the "structured refuge" for insect resistance management (IRM)

(Martinez and Reynolds, 2011), the U.S. EPA recently approved a seed mixture refuge

strategy (also called "refuge-in-the-bag" or "RIB") for planting pyramided Bt corn in the

northern U.S. Corn Belt where no cotton is planted (US-EPA, 2010; Onstad et al., 2011;

Matten et al., 2012).   The "RIB" strategy has not been approved in the southern U.S.

because of several technical concerns.   First, corn earworm is a major corn ear-feeding pest

that can overwinter in the U.S. southern region (Siegfried et al., 2000; Capinera, 2000; Siebert

et al., 2012).   Second, corn that has separate male and female flowering structures is a

cross-pollinating crop in which most pollination results from pollen dispersed by wind and

gravity (Chilcutt and Tabashnik, 2004; Burkness and Hutchison, 2012).   Pollen movement

from the surrounded Bt plants to the non-Bt refuge plants is a major concern for the use of the

"RIB" strategy where the target pests are primarily ear feeders, as in the case of corn

earworm (Burkness et al., 2011; Ives et al., 2011; Razze and Mason, 2012).   This cross

pollination could result in Bt expression in refuge kernels and thus may directly kill

susceptible refuge individuals or significantly delay their development if they feed on kernels.

In addition, pollen movement could also create sub-lethal exposure and promote selection for

resistance by increasing survival of the resistant heterozygotes or individuals carrying minor

resistance alleles (Wangila et al., 2013), especially when the Bt plants do not produce a 'high

dose' for the pest.   In addition, larval movement of more Bt-tolerant pest species (e.g., corn

earworm) among Bt and non-Bt plants may also create a favorable condition for resistance

development.   For example, movement of susceptible larvae from non-Bt refuge plants to Bt

plants in a "RIB" strategy could cause greater mortality to susceptible insects than in a structured refuge planting and thus result in a lower refuge population (Davis and Onstad, 2000). Furthermore, corn earworm is also a major target species of Bt cotton as well as pyramided Bt corn in the southern U.S. and thus there is a high potential for exposure to the Bt proteins in both Bt corn and Bt cotton across multiple corn earworm generations in this region (US-EPA, 2001).

In this study, multiple field trials were conducted to evaluate the occurrence and ear damage of corn earworm in different planting patterns of non-Bt and Bt corn containing Genuity® SmartStax™ traits. The objectives of the study were to determine 1) the preference of egg-laying of corn earworm among Bt and non-Bt plants, 2) the efficacy of pyramided Bt corn for control of corn earworm in mixed planting of Bt and non-Bt corn, and 3) if the non-Bt plants in "RIB" planting could serve an equivalent refuge function for corn earworm as structured refuge plantings.

4.2 Materials and Methods

4.2.1 Source of Bt and Non-Bt Corn Hybrids

A pyramided Bt corn hybrid, DKC 61-21 containing Genuity® SmartStax™ traits (Monsanto, St Louis, MO), and a genetically closely related non-Bt corn hybrid DKC 61-22 (Monsanto, St Louis, MO), were used in the field studies. Genuity® SmartStax™ corn contains six Bt genes as mentioned above, as well as two herbicide resistance traits glyphosate (*Roundup*) and glufosinate-ammonium (*Liberty*) (Gatehouse, 2008; Monsanto, 2012). Three of the six Bt genes, Cry1A.105, Cry2Ab2 and Cry1F, target above-ground lepidopteran pests including corn earworm. The other three Cry proteins, Cry3Bb1 and

Cry34/35Ab1, are for controlling the below-ground coleopteran rootworms, *Diabrotica* spp, and have no insecticidal activity against lepidopteran species (Monsanto, 2012). The non-Bt corn expresses both herbicide resistance traits but contains none of the Bt genes. Expression/non-expression of the Cry proteins in the corn hybrids was confirmed using an ELISA-based technique (EnviroLogix, Quantiplate[TM] kits, Portland, ME).

4.2.2 Planting Patterns and Experimental Designs

A total of six field trials were conducted in Franklin Parish near Winnsboro, Louisiana, USA in 2011 (two trials, hereafter referred to as Trial 2011-A and Trial 2011-B) and 2012 (four trials, hereafter referred to as Trial 2012-A, Trial 2012-B, Trial 2012-C and Trial 2012-D) (Table 4.1). Each trial consisted of three planting patterns of non-Bt and Bt corn plants. Each planting pattern contained three rows with nine plants in each row (a total of 27 plants) as described in Wangila et al. (2013). The three different planting patterns were: 1) pure stands of 27 Bt plants (pure Bt); 2) pure stands of 27 non-Bt plants (pure non-Bt), which was considered as a "structured refuge" planting; and 3) one non-Bt plant in the center surrounded by 26 Bt plants ("RIB"), simulating a 96:4% "RIB" planting (Wangila et al., 2013). The 96:4% rate of Bt and non-Bt corn was close to the currently used 95:5% "RIB" rate for pyramided Bt corn in the northern U.S. Corn Belt (US-EPA, 2010; Matten et al., 2012). The three planting patterns were arranged in a randomized complete block design with a total of seven blocks (replications) for each trial.

Field trials were planted at different times in 2011 and 2012. Natural infestations at the trial sites were high enough and thus no artificial infestations were used for all trials. For the trials in 2011, only ear damage (cm^2) data in the primary ears of the 27 plants of each plot

67

were recorded because most larvae had moved out of the ears when field samplings were conducted.   To assess corn earworm population dynamics and abundance during the test periods in 2012, plant damage and occurrence of corn earworm in the trial plots were monitored beginning in May-August depending on the planting dates to determine sampling time.   For the trials in 2012, data recorded were number of eggs per ear (primary ear only), number of larvae, larval growth stages, and ear damage (cm^2) by corn earworm.   Number of eggs per ear was checked by visual observation of the silks of 12-15 randomly selected primary ears per plot as well as all center refuge ears in the "RIB" plantings for the first three trials in 2012 (Trial 2012-A, Trial 2012-B, and Trial 2012-C).   The egg samplings were conducted at the peak of egg populations for each trial, while larval occurrence, larval stage, and ear damage were recorded for all 27 plants in a plot when the majority of the larvae on non-Bt plants were at the 3rd to 5th instar stages for all four trials in 2012.   The number of larvae recorded was the sum of the larvae observed on the primary and secondary ears on a plant.   Because it is difficult to measure the damaged area on the secondary ears and most

Table 4.1. Planting and sampling dates of six field trials in 2011 and 2012 for evaluation of occurrence and ear damage of *Helicoverpa zea* in three planting patterns of non-Bt and Bt corn plants containing Genuity® SmartStax™ traits.

Year	Trial	Planting date	Egg checking date	Larvae and ear damage checking date
2011	2011-A	3rd June	---	21th August
	2011-B	15th July	---	19th October
2012	2012-A	25th April	24th June	6th July
	2012-B	16th May	11th July	22th July
	2012-C	13th June	9th August	17th August
	2012-D	21th June	---	28th August

secondary ears are not harvestable, ear damage by corn earworm in secondary ears was not recorded in this study.

4.2.3 Data Analysis

Non-Bt plants (refuge) in the "RIB" planting pattern were considered as a separate treatment (Wangila et al., 2013). Data collected from the Bt plants in the "RIB" planting were separated from those recorded from the refuge plants and were considered as another treatment. Larval stages were converted to a development index: 1=1st instar, 2=2nd instar , …, 6=6th instar. Larval development index for the larvae found in each replication was calculated as the average of the development index. Data on number of eggs, number of larvae and their corresponding development index, and kernel damage (cm^2) were first transformed to ln (x +1) scale followed by one-way analysis of various (ANOVA) for each trial (SAS Institute, 2010). In addition, because the overall results were generally very consistent across the trials, data for each variable were combined across the six trials and the combined data were analyzed using mixed models with trial as a random factor (SAS Institute, 2010). Treatment means for each trial and the combined data were separated using Fisher's protected least significant difference (LSD) tests at $\alpha = 0.05$ level. Untransformed data are presented in the tables.

To better understand if planting patterns of Bt and non-Bt corn influenced the distributions of corn earworm, field distribution of eggs and larvae in each planting pattern in each trial was determined based on the dispersion index described in Davis (1994). The dispersion index was calculated by dividing the variance (s^2) by the mean (m) of the insect population for each planting pattern in a trial. To determine the field distribution of the insect population, the calculated dispersion index for a planting pattern in a trial was compared with the 95%

confidence interval (CI) of the value of a dispersion index = 1, which was estimated using the

formula: 95% CI $=1 \pm 2[2n/(n-1)^2]^{1/2}$, where n is the sample size.   If the calculated

dispersion index was less than $1-2[2n/(n-1)^2]^{1/2}$, the insect distribution in the field was

classified as uniform; if it was greater than $1+2[2n/(n-1)^2]^{1/2}$, the distribution was considered

aggregated; and if it fell within the 95% CI, the distribution was judged to be random (Patil

and Stiteler, 1974).

4.3 Results

4.3.1 Egg Occurrence of Corn Earworm in Mixed Plantings of Non-Bt and Bt Corn

Egg population of corn earworm was relatively high and consistent across the three trials

in which egg occurrence was investigated in this study.   Effect of treatment (plant

hybrid/planting pattern) on number of eggs per ear was not significant across all the three

trials as well as for the combined data (Table 4.2).   At the peak of egg occurrence, an

average of 2.4 - 6.7 eggs per ear was recorded in the primary ears of Bt and non-Bt plants

across the three trials.

Table 4.2. Egg occurrence (mean ± sem) of *Helicoverpa zea* in three planting patterns of non-Bt and Bt plants containing Genuity® SmartStax[TM] traits.

Planting pattern		2012-A	2012-B	2012-C	Combined
Pure Bt		2.43 ± 0.33 a	5.62 ± 0.53 a	3.27 ± 0.16 a	3.77 ± 0.37 a
Pure non-Bt		2.07 ± 0.19 a	6.00 ± 0.90 a	3.31 ± 0.17 a	3.79 ± 0.47 a
RIB	Non-Bt	2.61 ± 0.33 a	8.33 ± 1.84 a	3.14 ± 0.26 a	4.63 ± 0.82 a
	Bt	2.67 ± 0.42 a	6.69 ± 0.65 a	3.14 ± 0.30 a	4.15 ± 0.48 a
F-test	F-value	$F_{3,17} = 0.56$	$F_{3,17} = 1.57$	$F_{3,18} = 0.25$	$F_{3,58} = 1.02$
	P-value	0.65	0.23	0.86	0.39

[*]Means in a column followed by a different letter were significantly different (LSD test, α = 0.05).

4.3.2 Larval Occurrence and Development of Corn Earworm in Mixed Plantings of Non-Bt and Bt Corn

Larval population of corn earworm on non-Bt corn plants was also relatively high during the four trials in which larval occurrence were surveyed in the study. The overall larval occurrence in each treatment was also consistent across the four trials. Unlike the egg occurrence mentioned above, the effect of treatment on larval occurrence was significant for all four trials and the combined data (Table 4.3). Across the trials, an average of 3.78 larvae per plant was found on the ears of the refuge plants in the "RIB" planting, which was significantly greater ($P > 0.05$) than that (2.48/ear) observed in the pure stands of non-Bt plants (Table 4.3). Bt corn plants were effective against corn earworm. An average of only 0.46 larvae per plant was observed in the pure stands of Bt plants, which was not significantly ($P > 0.05$) different from that (0.51/plant) recorded on the Bt corn ears in the "RIB" planting (Table 4.3).

Table 4.3. Larval occurrence (mean ± sem) of *Helicoverpa zea* in three planting patterns of non-Bt and Bt plants containing Genuity® SmartStax™ traits.

Planting pattern		2012-A	2012-B	2012-C	2012-D	Combined
Pure Bt		0.04 ± 0.03 a	0.65 ± 0.07 a	0.34 ± 0.12a	0.82 ± 0.14 a	0.46 ± 0.07 a
Pure non-Bt		2.10 ± 0.17 b	3.09 ± 0.14b	2.72 ± 0.25b	2.00 ± 0.09 b	2.48 ± 0.12 b
RIB	Non-Bt	2.71 ± 0.57 b	6.00 ± 0.97 c	3.14 ± 0.40b	3.57 ± 0.48 c	3.78 ± 0.37 c
	Bt	0.12 ± 0.05 a	0.88 ± 0.12 a	0.25 ± 0.06a	0.79 ± 0.13 a	0.51 ± 0.08 a
F-test	F-value	$F_{3,18} = 59.12$	$F_{3,17} = 69.85$	$F_{3,18} = 69.39$	$F_{3,18} = 62.08$	$F_{3,80} = 185.83$
	P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[*]Means in a column followed by a different letter were significantly different (LSD test, α=0.05).

In general, larval development patterns of corn earworm in each treatment were also consistent across the four trials. Effect of treatment on the larval development index was significant for all four trials as well as for the combined data (Table 4.4). Results of ANOVA on the combined data showed that larvae on non-Bt refuge ears in the "RIB" plantings was significantly ($P < 0.05$) delayed relative to that in the pure stands of non-Bt plants (Table 4.4). Development index of larvae recovered from pure stands of non-Bt plants reached 3.92, while it was 3.55 for those larvae on non-Bt refuge ears in the "RIB" plantings. Larval development of corn earworm recovered from Bt corn ears was similar ($P > 0.05$) between pure Bt and "RIB" plantings and the larvae from both treatments was considerably ($P < 0.05$) delayed compared to those found on non-Bt corn plants. Most larvae recovered from Bt corn ears were still at the 2nd instar stage, with an average development index of 2.27 for the pure Bt corn and 2.36 for the "RIB" plantings (Table 4.4).

Table 4.4. Larval development index (mean ± sem) of *Helicoverpa zea* in three planting patterns of non-Bt and Bt plants containing Genuity® SmartStax™ traits.

Planting pattern		2012-A	2012-B	2012-C	2012-D	Combined
Pure Bt		2.30 ± 0.15 ab	2.11 ± 0.08 a	2.14 ± 0.05 a	2.56 ± 0.10 a	2.27 ± 0.10 a
Pure non-Bt		3.38 ± 0.07 b	4.16 ± 0.04 c	3.50 ± 0.15 b	4.64 ± 0.14 c	3.93 ± 0.11 c
RIB	Non-Bt	3.17 ± 0.29 b	3.49 ± 0.21 b	3.46 ± 0.23 b	4.07 ± 0.17 b	3.55 ± 0.13 b
	Bt	2.20 ± 0.22 a	2.28 ± 0.08 a	2.35 ± 0.12 a	2.60 ± 0.13 a	2.36 ± 0.07 a
F-test	F-value	$F_{3,13} = 7.35$	$F_{3,17} = 78.72$	$F_{3,18} = 26.68$	$F_{3,18} = 151.97$	$F_{3,75} = 113.34$
	P-value	0.004	<0.0001	<0.0001	<0.0001	<0.0001

[*]Means in a column followed by a different letter were significantly different (LSD test, α=0.05).
[#]Larval stages were converted to development index: 1=1st instar, 2=2nd instar, …, 6=6th instar. Larval development index for the larvae found in each replication was then calculated as the average of the development index.

Table 4.5. Ear damage (mean ± sem) of *Helicoverpa zea* in three planting patterns of non-Bt and Bt plants containing Genuity® SmartStax[TM] traits.

Planting pattern		2011-A	2011-B	2012-A	2012-B	2012-C	2012-D	Combined
Pure Bt		0.47 ± 0.16 a	0.61 ± 0.08 a	0.02 ± 0.01 a	0.17 ± 0.04 a	0.18 ± 0.05a	0.61 ± 0.06 a	0.34 ± 0.05 a
Pure non-Bt		1.91 ± 0.24 b	2.98 ± 0.10 b	6.02 ± 0.27 b	9.22 ± 0.49 b	9.89 ± 1.11b	11.39 ± 0.56 b	7.34 ± 0.61 b
RIB	Non-Bt	1.00 ± 0.65 a	4.43 ± 0.48 c	5.00 ± 0.87 b	10.33 ± 1.15 b	9.86 ± 1.71b	15.57 ± 0.81 c	7.63 ± 0.85 b
	Bt	0.64 ± 0.20 a	0.51 ± 0.11 a	0.05 ± 0.03 a	0.29 ± 0.06 a	0.17 ± 0.05a	0.56 ± 0.04 a	0.37± 0.05 a
F-test	F-value	$F_{3,17}$ = 3.47	$F_{3,15}$ = 67.00	$F_{3,18}$ = 113.69	$F_{3,17}$ = 348.21	$F_{3,18}$ = 180.35	$F_{3,18}$ = 1323.68	$F_{3,118}$=182.60
	P-value	0.04	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[*]Means in a column followed by a different letter were significantly different (LSD test, α=0.05).

4.3.3 Ear Damage by Corn Earworm in Different Planting Patterns of Non-Bt and Bt Corn

Effect of treatment on ear damage area by corn earworm was significant for all six trials and the combined data.   A few variations in ear damage patterns among the six trials were observed, but the overall results were, in general, consistent across the six trials.   Based on the combined data, an average damage area of 7.63 cm^2/ ear was recorded from the non-Bt refuge ears in "RIB" plantings, which was similar ($P > 0.05$) to that (7.34 cm^2/ ear) observed in the pure stands of non-Bt corn.   Compared to the non-Bt corn plants, ear damage of Bt corn plants by corn earworm was significantly reduced with an average of 0.34 cm^2/ ear on ears of pure Bt plantings and 0.37 cm^2/ ear on ears of 'RIB' plantings.   The small difference in the ear damage observed on Bt corn ears was not significant ($P > 0.05$) between the pure Bt and "RIB" plantings (Table 4.5).

4.3.4 Egg and Larval Distribution of Corn Earworm in Three Planting Patterns of Non-Bt and Bt Corn

Based on the dispersion index (variance / mean), eggs were distributed randomly among plants in the pure stands of Bt and "RIB" plantings across all three trials evaluated in 2012 (Table 4.6).   In the pure stands of non-Bt plants, eggs were distributed uniformly in Trial 2012-A and Trial 2012-C, while egg population in Trial 2012-B fitted an aggregated distribution.   However, the dispersion index (1.376) observed in the pure non-Bt planting in Trial 2012-B only slightly departed from the upper limit (1.312) of the 95% CI for a random distribution.

Larvae of corn earworm in the pure stands of non-Bt corn plants were also distributed either randomly (Trial 2012-A and Trial 2012-C) or uniformly (Trial 2012-B and Trial 2012-D) (Table 4.6).   In contrast, larvae in the pure stands of Bt plants and "RIB" plantings

74

appeared to be in an "aggregated" distribution across the four trials except for the pure stands

of Bt plants in Trial 2012-A which fitted a random distribution (Table 4.6).

Table 4.6. Egg and larval distribution of *Helicoverpa zea* in three planting patterns of non-Bt and Bt plants containing Genuity® SmartStax[TM] traits.

Trial	Plant pattern	Eggs		Larvae	
		Dispersion index (s^2/m)	Distribution	Dispersion index (s^2/m)	Distribution
	Pure Bt	0.944	Random	0.959	Random
2012-A	Pure non-Bt	0.630	Uniform	1.106	Random
	RIB	0.810	Random	1.967	Aggregated
	Pure Bt	1.080	Random	1.407	Aggregated
2012-B	Pure non-Bt	1.376	Aggregated	0.591	Uniform
	RIB	1.298	Random	2.573	Aggregated
	Pure Bt	0.788	Random	2.107	Aggregated
2012-C	Pure non-Bt	0.546	Uniform	0.981	Random
	RIB	0.842	Random	1.807	Aggregated
	Pure Bt	---	---	1.382	Aggregated
2012-D	Pure non-Bt	---	---	0.479	Uniform
	RIB	---	---	1.423	Aggregated

[*] 95% confident interval (CI) of the value of dispersion index=1, which was calculated using the formula: 95% CI $=1 \pm 2[2n/(n-1)^2]^{1/2}$.   Dispersion index $< 1-2[2n/(n-1)^2]^{1/2}$ means that the distribution is uniform, $> 1-2[2n/(n-1)^2]^{1/2}$ means that the distribution is aggregated, and a value in between denotes that the distribution is random.

4.4 Discussion

In this study, egg-laying of corn earworm among Bt and non-Bt corn ears was investigated in three of the six field trials and there were no significant differences in the number of eggs laid on the silks of Bt and non-Bt plants among the three planting patterns across all three trials as well as for the combined data.   Analysis of the dispersion index also showed that almost all egg populations were distributed either randomly or uniformly among plants in the three planting patterns and across the three trials.   The results suggest that females of corn earworm have no egg-laying preference between Bt and non-Bt plants.   The phenomenon of females indiscriminately laying eggs among Bt and non-Bt plants has been reported in the European corn borer, *Ostrinia nubilalis* (Hübner), pink bollworm, *Pectinophora gossypiella*, and cotton bollworm, *Helicoverpa armigera* (Orr and Landis, 1997; Hellmich et al., 1999; Hutchison et al., 2010; Liu et al., 2002; Dhillon and Sharma, 2013). This biological characteristic is critically important in pest management because natural pest populations of a target species could be greatly reduced when Bt corn is widely planted in a region, resulting in less damage to non-Bt corn plants in that region (Hutchison et al., 2010).

Larval occurrence of corn earworm at the peak of the 3rd -5th instar stages was evaluated on Bt and non-Bt corn ears in all four field trials that were conducted in 2012.   Larval populations of corn earworm on Bt corn ears in both the pure stands of Bt corn and "RIB" plantings were significantly less than the populations on non-Bt corn ears in all four trials and for the combined data.   In addition, the development of larvae feeding on Bt corn ears, if they survived, was delayed compared to on non-Bt corn ears.   This developmental delay was significant in three of the four trials and the combined data.   In the Trial 2012-A where

76

statistical significance was not detected, larvae recovered from Bt corn ears were numerically

delayed approximately one-instar.   Furthermore, the results of all the six trials in this study

as well as the combined data showed considerably less damages on Bt corn ears than on

non-Bt plants.   Collectively, data from this study demonstrate that the pyramided Genuity[®]

SmartStax[TM] corn was effective in controlling corn earworm and protecting from ear damage.

The current study also found no significant differences in larval occurrence, larval

development, or ear damage on Bt corn ears between "RIB" and pure stands of Bt corn across

all six trials and the combined data analysis.   The results suggest that the Genuity[®]

SmartStax[TM] corn plants are equally effective against corn earworm in pure stands of Bt corn

and "RIB" plantings.   In a similar field design, Wangila et al. (2013) also reported that

Genuity[®] SmartStax[TM] was equally effective in pure stands of Bt corn and "RIB" plantings

for controlling the sugarcane borer, *Diatraea saccharalis* (F.), a major target of Bt corn in the

mid-south region of the United States.   Carroll et al. (2013) also found no significant

differences in tunnel number or tunnel length caused by southwestern corn borer, *Diatraea*

grandiosella Dyar, between pure stands of MON 89034 corn and mixed plantings of MON

89034 and non-Bt corn.

As observed in the egg distributions, larvae of corn earworm at the peak of the 3rd to 5th

instar stages were distributed either randomly or uniformly in the pure stands of non-Bt plants,

suggesting that larval movement among plants in the early growth stages may be limited in

this case.   However, corn earworm larvae distributed in an "aggregated" fashion in pure

stands of Bt and "RIB" planting patterns.   The non-random distribution of corn earworm

larvae in the pure stands of Bt plants could be due to uneven expression of Bt proteins among

individual plants and/or more larval movement than with pure stands of non-Bt plants (Halcomb et al., 2000; Chilcutt, 2006; Razze and Mason, 2012). Elevated larval movement on Bt plants relative to non-Bt plants has been observed in European corn borer (Razze and Mason, 2012). In the "RIB" planting, another possible reason of the "aggregated distribution" is the considerably higher survival rates of larvae on ears of the non-Bt refuge plants. More studies are needed to understand the implications of the uneven larval survival among Bt plants for resistance management.

Larval occurrence and ear damage of corn earworm on non-Bt corn refuge plants in the "RIB" planting in the current study were either similar to or greater than that observed in pure stands of non-Bt plants across all six trials during the two years. The results suggest that pollen movement from Bt plants to non-Bt refuge plants did not result in a significant reduction in larval populations of corn earworm at the early larval stages (e.g $\leq 5^{th}$ instars). However, the effect of Bt protein contamination in providing refuge populations in a "RIB" strategy might not be measured accurately if the analysis is relied on only the data obtained from the open field observations. To determine if a "RIB" planting could provide the expected susceptible refuge population of a target species like corn earworm for IRM, it is necessary to know the effect of Bt protein contamination in refuge ears on the biology of the full life cycle of corn earworm. In corn fields, larvae of corn earworm feed on corn ears or plants and mature larvae exit the ears or plants and then drop to the ground and pupate in the soil (Capinera, 2000). Therefore, a full assessment of the impacts of cross- pollination on the full life cycle of corn earworm will be challenging under field conditions. Several laboratory studies have shown that exposure to sub-lethal doses of Bt toxin could result in

prolonged larval and prepupal development, smaller pupae, and reduced fecundity of corn

earworm (Horner and Dively, 2003; Horner et al., 2003; Johnson and Gould, 1992).   In the

current study, developmental delays of corn earworm larvae on refuge ears in the "RIB"

plantings relative to larvae found on ears of pure stands of non-Bt corn were significant in

two of the four trials as well as for the combined data.   Across the four trials in the

combined data, the average development index of larvae recovered from the pure stands of

non-Bt corn plants was 3.93, compared to 3.55 for those found on "RIB" refuge ears.   Given

these results, further studies are warranted to clarify the effects of cross-pollination in "RIB"

plantings on the full life cycle of corn earworm.

Nevertheless, the number of corn earworm larvae recovered from "RIB" refuge plants

appeared to be more than that found in pure stands of non-Bt plants.   The difference was

significant for the combined analysis and for two of the four trials in 2012 (Trials 2012-B

&2012-D), which also corresponded to the two trials where larval development on refuge

ears was significantly delayed compared to on pure non-Bt corn ears.   The reduced number

of larvae in the pure stands of non-Bt plants relative to that on the refuge plants in "RIB"

plantings could be due to several reasons including larval cannibalism of corn earworm.

Studies have shown that cannibalism has a major impact on the population dynamics of corn

earworm on corn ears, and can reduce population size (Stinner et al., 1977).   As larvae of

corn earworm develop, the intensity of cannibalism increases (Polis, 1981; Joyner and Gould,

1985; Horner and Dively, 2003; Chilcutt, 2006).   As observed in this study, larvae feeding

on ears of pure stands of non-Bt plants were larger than those recovered from "RIB" refuge

plants, especially in Trial 2012-B and Trial 2012-D.   Thus it is possible that the intensity of

cannibalism was greater in the pure stands of non-Bt plants than on the "RIB" refuge ears

during the test periods.   Another reason of the greater larval numbers on "RIB" refuge ears

might be due to larval movement.   Wangila et al. (2013) showed that, in mixed planting

patterns of Bt and non-Bt corn, larvae of sugarcane borer could move between Bt and non-Bt

plants.   Such movement could be elevated in "RIB" plantings (Razze and Mason 2012).

Therefore, it is possible that larvae on Bt plants are more likely to move and end up on

non-Bt plants in a mixed "RIB" planting.   However, larvae of different insects may display

distinctive behaviors.   More research is needed to determine the movement behavior of corn

earworm under the "RIB" scenario so that science-based IRM strategies can be developed for

the sustainable use of Bt corn technologies

4.5 References

Burkness, E.C., Dively, G., Patton, T., Morey, A.C., Hutchison, W.D., 2010. Novel Vip3A
    Bacillus thuringiensis (Bt) maize approaches high-dose efficacy against *Helicoverpa zea*
    (Lepidoptera: Noctuidae) under field conditions. GM Crop. 1, 1-7.

Burkness, E.C., O'Rourke, P.K., Hutchison, W.D., 2011. Cross-pollination of nontransgenic
    corn ears with transgenic Bt corn: efficacy against lepidopteran pests and implications
    for resistance management. J. Econ. Entomol. 104, 1476-1479.

Burkness, E.C., Hutchison, W.D., 2012. Bt pollen dispersal and Bt kernel mosaics: integrity
    of non-Bt refugia for lepidopteran resistance management in maize. J. Econ. Entomol.
    105, 1477-1870.

Capinera, J.L., 2000. Corn Earworm, *Helicoverpa* (=*Heliothis*) *zea* (Boddie) (Lepidoptera:
    Noctuidae). Florida Cooperative Extension Service, Institute of Food and Agricultural
    Sciences, University of Florida. EENY-145 (IN302).

Carroll, M.W., Head, G., Caprio, M., Stork, L., 2013. Theoretical and empirical assessment of
    a seed mix refuge in corn for southwestern corn borer. Crop Protect. 49, 58-65.

Chilcutt, C.F., 2006. Cannibalism of *Helicoverpa zea* (Lepidoptera: Noctuidae) from *Bacillus
    thuringiensis* (Bt) transgenic corn versus non-Bt corn. J. Econ. Entomol. 99, 728-732.

Chilcutt, C.F., Tabashnik, B.E., 2004. Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maize. Proc. Natl. Acad. Sci. U.S.A. 101, 7526-7529.

Davis, P.M., 1994. Statistics for describing populations, pp.33-54. In L. P. Pedigo and G. D. Buntin(eds.). Handbook of Sampling Methods for Arthropods in Agriculture. CRC Press. London.

Davis, P.M., Onstad, D.W., 2000. Seed mixtures as a resistance management strategy for European corn borers (Lepidoptera: Crambidae) infesting transgenic corn expressing Cry1Ab protein. J. Econ. Entomol.: 93, 937-948.

Dhillon, M.K., Sharma, H.C., 2013. Comparative studies on the effects of Bt-transgenic and nontransgenic cotton on arthropod diversity, seedcotton yield and bollworms control. J. Environ. Biol. 34, 67-73.

FIFRA Scientific Advisory Panel, 1998. Report of Subpanel on *Bacillus thuringiensis* (Bt) plant-pesticides and resistance management. EPA SAP Report. Available at: http://www.mindfully.org/GE/FIFRA-SAP-Bt.htm.

Gatehouse, J.A., 2008. Biotechnological prospects for engineering insect-resistant plants. Plant Physiol. 146, 881-887.

Halcomb, J.L., Benedict, J.H., Cook, B., Ring, D.R., Correa, J.C., 2000. Feeding behavior of bollworm and tobacco budworm (Lepidoptera: Noctuidae) larvae in mixed stands of nontransgenic and transgenic cotton expressing an insecticidal protein. J. Econ. Entomol. 93, 1300-1307.

Hellmich, R.L., Higgins, L.S., Witkowski, J.F., Campbell, J.E., Lewis, L.C., 1999. Oviposition by European corn borer (Lepidoptera: Crambidae) in response to various transgenic corn events. J. Econ. Entomol. 92, 1014-1020.

Horner, T.A., Dively, G.P., 2003. Effects of MON810 Bt field corn on *Helicoverpa zea* (Lepidoptera: Noctuidae) cannibalism and its implication to resistance development. J. Econ. Entomol. 96, 931-934.

Horner, T.A., Dively, G.P., Herbert, D.A., 2003. Development, survival, and fitness performance of *Helicoverpa zea* (Lepidoptera: Noctuidae) in MON810 Bt field corn. J. Econ. Entomol. 96, 914-924.

Hutchison, W.D., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S.J., Abrahamson, M., Hamilton, K.L., Steffey, K.L., Gray, M.E., et al., 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt mazie growers. Science, 222-225.

Ives, A.R., Glaum, P.R., Ziebarth, N.L., Andow, D.A., 2011. The evolution of resistance to two-toxin pyramided transgenic crops. Ecol. App. 21, 503-515.

Johnson, M.T., Gould, F., 1992. Interaction of genetically engineered host plant resistance and natural enemies of *Heliothis virescens* (Lepidoptera: Noctuidae) in tobacco. Environ. Entomol. 21, 586-597.

Joyner, K., Gould, F., 1985. Developmental consequences of cannibalism in *Heliothis zea* (Lepidoptera: Noctuidae). Ann. Entomol. Soc. Am. 78, 24-28.

Liu, Y.B., Tabashnik, B.E., Dennehy, T.J., Carrière, Y., Sims, M.A., Meyer, S.K., 2002. Oviposition on and mining in bolls of Bt and non-Bt cotton by resistant and susceptible pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 95, 143-148.

Martinez, J., Reynolds, A., 2011. Updated EPA review of ABSTC's 2010 corn insect resistance management compliance assurance. Available at: http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0922-0014.

Matten, S.R., Frederick, R.J., Reynolds, A.H., 2012. United States Environmental Protection Agency insect resistance management programs for plant-incorporated protectants and use of simulation modeling. pp 175-267. *In:* C.A. Wozniak, A. McHughen (eds), "Regulation of agricultural biotechnology: The United States and Canada". Springer.

Monsanto, 2012. IRM grower guide: insect resistance management for U.S. corn and cotton-growing areas. Available at: http://www.monsanto.com/products/Pages/insect-resistance-management.aspx.

Onstad, D.W., Mitchell, P.D., Hurley, T.M., Lundgren, J.G., Porter, R.P., Krupke, C.H., Spencer, J,L., DiFonzo, C.D., Baute, T.S., Hellmich, R.L., Buschman, L.L., Hutchison, W.D., Tooker, J.F., 2011. Seeds of change: corn seed mixtures for resistance management and IPM. J. Econ. Entomol. 104, 343-352.

Orr, D.B., Landis, D.A., 1997. Oviposition of European corn borer (Lepidoptera: Pyralidae) and impact of natural enemy populations in transgenic versus isogenic corn. J. Econ. Entomol. 90, 905-909.

Patil, G.P., Stiteler, W.M., 1974.Concepts of aggregation and their quantification: A critical review with some new results and applications. Res. Popul.Ecol. 15, 238-254.

Polis, G.A., 1981. The evolution and dynamics of intraspecific predation. Annu. Rev. Ecol. Syst. 12, 225-251.

Razze, J.M., Mason, C.E., 2012. Dispersal behavior of neonate European corn borer (Lepidoptera: Noctuidae) on Bt corn. J. Econ. Entomol. 105, 1214-1223.

Roush, R.T., 1998. Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? *Phil Trans R Soc Lond B* 353, 1777-1786.

Siebert, M.W., Nolting, S.P., Hendrix, W., Dhavala, S., Craig, C., Leonard, B.R., Stewart, S.D., All, J., Musser, Buntin, F.R., Samuel, L., 2012. Evaluation of corn hybrids expressing Cry1F, Cry1A.105, Cry2Ab2, Cry34Ab1/Cry35Ab1, and Cry3Bb1 against southern United States insect pests. J. Econ. Entomol. 105, 1825-1834.

Siegfried, B.D., Spencer, T., Nearman, J., 2000. Baseline susceptibility of the corn earworm (Lepidoptera: Noctuidae) to the Cry1Ab toxin from *Bacillus thuringiensis*. J. Econ. Entomol. 93, 1265-1268.

Stinner, R.E., Jones, J.W., Tuttle, C., Caron, R.E., 1997. Population mortality and cyclicity as affected by intraspecific competition. Can. Entomol. 109, 879-890.

SAS Institute, 2010. SAS/ STAT User's Third Edition, SAS Institute Inc, Cary, NC.

Syngenta, 2012. Corn trait Information: Agrisure Viptera™ 3111. Available at: http://www.syngenta.com/country/us/en/Seeds/Traits/CornTraits/Pages/content_authoring_Agrisurevip3111.aspx.

US-EPA (United States Environmental Protection Agency), 2001. Biopesticide registration action document: *Bacillus thuringiensis* plant-incorporated protectants. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm.

US-EPA (United States Environmental Protection Agency), 2010. Terms and conditions for Bt corn registrations 30 Sept 2010. Office of Pesticide Programs, Washington, DC. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/bt-corn-terms-conditions.pdf.

Wangila, D.S., Leonard, B.R., Ghimire, M.N., Bai, Y., Zhang, L., Yang, Y., Emfinger, K.D., Head, G.P., Yang, F., Niu, Y., Huang, F., 2013. Occurrence and larval movement of Diatraea saccharalis (Lepidoptera: Crambidae) in seed mixes of non-Bt and Bt pyramid corn. Pest Manag. Sci. 69, 1163-1172.

Yang, F., Huang, F., Qureshi, J.A., Leonard, B.R., Niu, Y., Zhang, L., Wangila, D.S., 2013. Susceptibility of Louisiana and Florida populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to transgenic Agrisure® Viptera™ 3111 corn. Crop Protect. 50, 37-39.

Zhao, J.Z., Cao, J., Li, Y., Collins, H.L., Roush, R.T., Earle, E.D., Shelton, A.M., 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat. Biotechnol. 21, 1493-1497.

CHAPTER 5. BT PROTEIN CONTAMINATION AND PERFORMANCE OF AGRISURE® VIPTERA™ 3111 CORN AGAINST *HELICOVERPA ZEA* (LEPIDOPTERA: NOCTUIDAE) IN SEED MIXED PLANTINGS[4]

5.1 Introduction

Since it was first commercialized in 1996, transgenic corn expressing *Bacillus thuringiensis* (Bt) proteins has been widely planted in the U.S. and many other countries in the world (Huang et al., 2011; James, 2013).   In 2013, 76% of the U.S. field corn was planted to Bt corn (USDA-NASS, 2013).   Bt corn products, in general, have provided effective control of targeted pest populations.   However, the extensive use of Bt corn imposes a high selection pressure on target pest populations that can result in resistance development (Matten et al., 2008; Storer et al., 2010; Van-Rensburg, 2007; Dhurua and Gujar, 2011; Gassmann et al., 2011).   To delay resistance development, an insect resistance management (IRM) plan, also known as the "high-dose/refuge" strategy has been adopted for planting Bt corn in the U.S. (Ostlie et al., 1997; US-EPA, 2001, Matten et al., 2012).

Recently, a gene pyramiding strategy has been used to develop transgenic corn containing two or more Bt proteins that are dissimilar in mode of action but effective against a same target pest (Moar and Anilkumar, 2007; Monsanto, 2012; Syngenta, 2012).   Since 2010, such Bt corn hybrids expressing pyramided Bt genes (e.g. Agrisure® Viptera™ 3111) have been commercially grown for controlling both above- and below-ground corn insect pests in the U.S. (US-EPA, 2009; 2010).   Compared to the 1st generation single-gene Bt corn (e.g. YieldGard ® corn borer), pyramided Bt corn is believed to be more effective against some noctuid species and be able to delay resistance development (Roush, 1998; Zhao et al.,

---

[4] Submitted for publication in Crop Protection

2003; Ives et al., 2011).   Because of the availability of the pyramided Bt corn products, the

U.S. Environmental Protection Agency (US-EPA) has approved to use a seed mixture refuge

strategy (also called "refuge-in-the-bag" or "RIB") for planting pyramided Bt corn hybrids in

the U.S. Corn Belt where no cotton is planted (Onstad et al., 2011; Matten et al., 2012).

Within the RIB scenario, a portion of non-Bt corn seeds is mixed with Bt corn seeds in each

bag by seed providers before being sold to farmers (Matten et al., 2012).   Currently, the

approved seed mixture in the U.S. is at a rate of 95: 5% Bt and non-Bt corn seeds (Reynolds,

2008; Matten et al., 2012; Monsanto, 2012).   One of the major concerns in use of a RIB

strategy is the cross-pollination property of corn hybrids that will result in Bt protein

contamination in refuge corn kernels in the seed mixed plantings.   The Bt protein

contamination in refuge ears could negatively affect susceptible refuge insect populations (e.g.

causing a higher mortality), especially for kernel-feeders, such as the corn earworm,

Helicoverpa zea (Boddie).   Corn earworm is a major target of pyramided Bt corn in the U.S.

(Monsanto, 2012; Syngenta, 2012).

In a previous study, we evaluated the occurrences, distribution, and ear damage of corn

earworm in mixed plantings of non-Bt and Bt corn containing Genuity® SmartStax™ trait

(Yang et al., 2014a).   Genuity® SmartStax™ is one of the most widely planted pyramided

Bt corn trait in the U.S.   The field study by Yang et al. (2014a) demonstrates that Genuity®

SmartStax™ corn products are equally effective against corn earworm in pure stands of Bt

corn and RIB plantings.   In this study, we assessed another commonly used pyramided Bt

corn trait, Agrisure® Viptera™ 3111, for controlling corn earworm in both pure stand and

RIB plantings.   The main objectives of the current study were to determine: 1) Bt protein

contamination in kernels of refuge ears due to cross-pollination in RIB plantings, and 2)

performance of the pyramided Bt corn products containing Agrisure® Viptera™ 3111 trait

against corn earworm.   In addition, by comparing the data from the previous study by Yang

et al. (2014a), to evaluate if the biological parameters such as occurrence and distribution of

corn earworm in RIB plantings were consistent among different traits.

5. 2 Materials and Methods

5.2.1 Bt and Non-Bt Corn Hybrids

A pyramided Bt corn hybrid, NK N78N-3111 (Syngenta, Minnetonka, MN) containing

Agrisure® Viptera™3111 trait (hereafter called Viptera 3111), and a genetically closely

related non-Bt corn hybrid Agrisure® NK N78N-GT (Syngenta, Minnetonka, MN), were used

in the field study.   Viptera 3111 corn contains two Bt genes, Vip3A and Cry1Ab, for

controlling above-ground lepidopteran species including corn earworm, and one gene,

mCry3A, for managing below-ground rootworms, *Diabrotica* spp. (Difonzo and Collen,

2012).   Vip3A is an exotoxin produced during the vegetative growth stage of Bt bacteria and

it shares no sequence homology with any known Bt Cry proteins (Kurtz, 2010).   The

mCry3A protein is non-toxic to lepidopteran species.

5.2.2 Experimental Design and Field Sampling

A total of eight field trials were conducted in Franklin Parish near Winnsboro, Louisiana,

U.S. in 2011(four trials, hereafter referred as Trial 2011-I, Trial 2011-II, Trial 2011-III, and

Trial 2011-IV) and 2012 (four trials, hereafter referred to as Trial 2012-I, Trial 2012-II, Trial

2012-III, and Trial 2012-IV) (Table 5.1).   Each trial consisted of three planting patterns of

non-Bt and Bt corn plants as described in Yang et al. (2014a).   Each planting pattern

included three rows with nine plants in each row (a total of 27 plants).   The three planting

patterns were 1) pure stands of 27 Bt plants; 2) pure stands of 27 non-Bt plants, which was

considered as a "structured refuge" planting; and 3) one non-Bt plant in the center surrounded

by 26 Bt plants (a 96:4% RIB).   The three planting patterns were arranged in a randomized

complete block design with a total of 5-7 replications for each trial (Table 5.1).   Different

planting patterns in a block were separated by one row distance (e.g. 1 m), and the distance

between blocks was 3- 4.5 m.

Table 5.1. Plantings and samplings of eight field trials conducted in 2011 and 2012 for assessment of Bt protein contamination in refuge ears and performance of Agrisure® Viptera™ 3111 corn against *Helicoverpa zea* in three planting patterns.

Year	Trial	Planting date	No. replications	Egg checking date	Larvae and ear damage evaluation date
2011	2011-I	28th February	5	-----	21th June
	2011-II	4th April	5	-----	26th June
	2011-III	3rd August	7	-----	21th August
	2011-IV	15th July	5	-----	19th October
2012	2012-I	25th April	7	24th June	6th July
	2012-II	16th May	7	11th July	22th July
	2012-III	13th June	7	9th August	17th August
	2012-IV	21th June	7	-----	28th August

Natural infestations of corn earworm were used for all eight trials in the two years.   For

the four trials that were conducted in 2011, only ear damage area (cm^2) of the primary ears

was recorded because most larvae had moved out from the ears when field samplings were

conducted for these trials.   For the trials in 2012, data recorded were the number of eggs per

ear (primary ear only), number of larvae, larval growth stages, and ear damage area (cm^2). As described in Yang et al. (2014a), number of eggs per ear was checked at the peak of egg populations by visual observation of the silks of 12-15 randomly selected primary ears of each plot for the first three trials in 2012 (Trial 2012-I, Trial 2012-II, and Trial 2012-III), while insect occurrence, larval stage, and ear damage were recorded for all four trials and for all 27 plants in each plot.   The number of larvae recorded was the total larvae observed on both primary and secondary (if existed) ears of a plant, while ear damage area was measured based on the primary ears only.

5.2.3 Qualitative Analysis of Bt Protein Expression in Individual F_2 Kernels

To measure Bt protein contamination in refuge kernels, primary ears of non-Bt refuge plants in RIB plantings were sampled for protein expression assays right after the above mentioned field samplings were completed for the first two trials in 2012 (Trials 2012-I and 2012-II).   At the same time, eight primary ears were also randomly collected from the pure stand of Bt corn plantings for each of the two trials.   To assay Bt protein expression, five (for pure Bt corn planting) and ten (for refuge ears of the RIB planting) individual kernels were sampled from the top to the bottom of each ear.   The presence/absence of Vip3A and Cry1Ab proteins in individually sampled kernels was examined using an ELISA-based technique according to the protocol of Quantiplate[TM] test strip kits (EnviroLogix, Portland, ME, USA) (Fig. 5.1).   Expression of the protein mCry3A was not performed because it is not toxic to lepidopteran species.   Protein expression of individual kernels of pure non-Bt corn planting was not performed but presence/absence of Bt proteins in Bt and non-Bt plants was validated using the same ELISA method as described in Wangila et al. (2012).
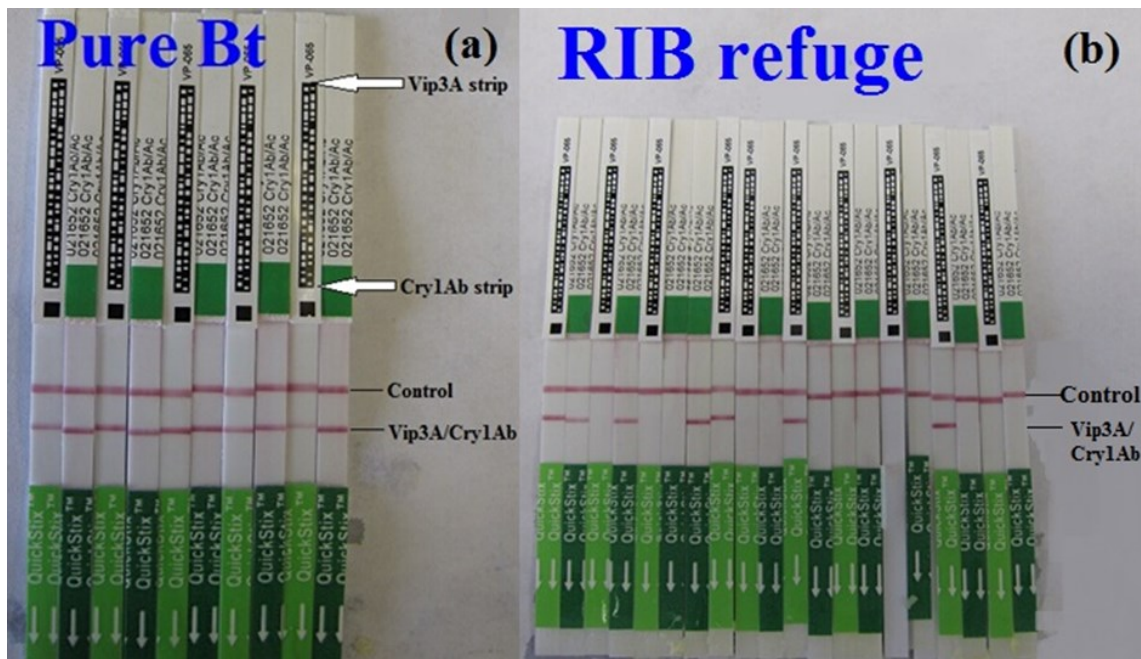
Fig. 5.1. A diagram showing kernel protein expression test. (a) Kernels from pure Bt ears; (b) Kernels from RIB refuge non-Bt ears. The black strips were used for testing the expression of Vip3A protein, and the green strips were used for testing the expression of Cry1Ab protein. Every adjacent two strips represented the results of protein expression test of Vip3A and Cry1Ab in a single kernel.

5.2.4 Data Analysis

Percentages of kernels containing one or more Bt proteins in ears removed from pure Bt corn plantings and ears from refuge plants in RIB plantings was calculated based on the number of kernels expressing the Bt proteins divided by the total kernels assayed. χ^2-tests were used to analyze if the two genes (Vip3A and Cry1Ab) in Viptera 3111 that target lepidopteran species were segregated independently based on presence/absence of the protein expression in kernels of the refuge ears.

In analysis of the data on larval occurrence, development, and ear damage, data recorded from Bt plants and non-Bt plants (refuge) in RIB plantings were separated and defined as two different treatments (Wangila et al., 2013). Recorded larval stages were transformed to developmental index: 1=1st instar, 2=2nd instar , …, 6=6th instar (Yang et al., 2014a). Data on

number of eggs, number of larvae and their corresponding development index, and kernel damage area (cm^2) were transformed to log (x +1) scale for normal distribution (Zar, 1984). The transformed data were then analyzed with one-way analysis of variance for each trial (SAS Institute, 2010). In addition, data for each variable were also pooled across all trials in which the corresponding variable was measured and the pooled data were then analyzed using mixed models with trial as a random factor (SAS Institute, 2010). Treatment means for each trial as well as the pooled analysis were separated using Tukey's HSD (honest significant difference) test at $\alpha = 0.05$ level. Non-transformed data are presented in the tables and figures. In addition, egg and larval distribution of corn earworm in each planting pattern for each trial was classified as uniform, random, or aggregated based on the corresponding dispersion indexes that were calculated using the same methods as described in Yang et al. (2014a).

5.3 Results

5.3.1 Protein Expression in Corn Kernels

ELISA tests showed that all 80 kernels removed from the 16 ears of pure stand of Bt plants in the two trials (Trials 2012-I and 2012-II) contained both Vip3A and Cry1Ab proteins (Table 5.2). Non-Bt expression in non-Bt plants was also confirmed with the ELISA tests. A high portion of kernels from the non-Bt refuge plants in RIB plantings were contaminated to express Bt proteins. Protein expression in refuge kernels was generally consistent for both proteins and between the two trials in which the Bt protein expressions were measured. Across the two trials, an average of 42.9% refuge kernels expressed either Vip3A or Cry1Ab and 24.2% expressed both proteins (Table 5.2). As a result, an average of 38.4% refuge kernels in the two trials didn't express Vip3A or Cry1Ab protein. Based on the data

Table 5.2. Percentage of kernels expressing one or two Bt proteins in the pure stand of Bt and RIB plantings and the corresponding χ^2-tests for independent segregation of the two Bt genes, Cry1Ab and Vip3A, in Agrisure® Viptera™ 3111corn.

Treatment	Pure stand of Bt corn (%)	RIB refuge ear (Trial 2012-I)			RIB refuge ear (Trial 2012-II)		
		Observed (mean ± sem %)	Expected (%)	χ^2	Observed (mean ± sem %)	Expected (%)	χ^2
Vip3A	100	38.3 ± 6.0	---	---	53.3 ± 5.6	---	---
Cry1Ab	100	43.3 ± 8.0	---	---	36.7 ± 6.2	---	---
Vip3A+Cry1Ab	100	21.7 ± 8.3	16.6	1.88	26.7± 4.9	19.6	3.20
Negative	0	40.0 ± 5.8	---	---	36.7 ± 4.9	---	---

Note: For pure stand of Bt corn planting, 40 kernels of 8 randomly sampled ears (5 kernels/ear) were individually tested for the Bt protein expressions using the ELISA method in each trial, while for RIB planting, 60 kernels of 6 refuge ears (10 kernels/ear) were individually examined for each trial.

recorded in refuge kernels, χ^2 tests showed that the observed frequency of presence/absence of the two proteins, Vip3A and Cry1Ab, fitted ($P > 0.05$) the assumption of independent segregation for both trials (Table 5.2).
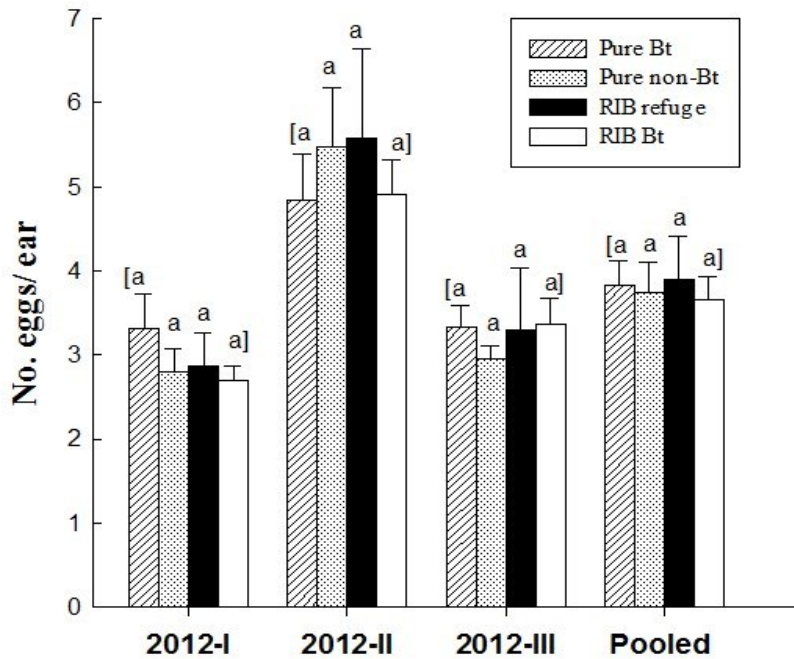
Fig. 5.2. Egg occurrence (mean ± sem) of *Helicoverpa zea* in three planting patterns of non-Bt and Bt plants containing Agrisure® Viptera™ 3111 traits. Means in each trial and combined analysis followed by the same letter were not significantly different (Tukey's HSD test, α = 0.05).

5.3.2 Occurrence and Distribution of Corn Earworm Eggs in Three Planting Patterns of Non-Bt and Bt Corn Containing Viptera 3111 Traits

Natural egg populations of corn earworm were relatively high and consistent across the three trials in which egg occurrence was investigated. Effect of corn hybrid/planting pattern on egg occurrence was not significant across all three trials ($F \leq 0.65$; df = 3, 18; $P \geq 0.59$) as well as for the pooled data ($F = 0.18$; df = 3, 60; $P = 0.91$). During the peaks of egg oviposition, an average of 2.9 - 5.2 eggs per ear was observed in the primary ears of Bt and non-Bt plants across the three planting patterns and the three trials (Fig. 5.2).

92

In addition, it is apparent that corn hybrids (Bt and non-Bt corn) and planting patterns had no significant effects on egg distributions.   In the corn field, eggs of corn earworm were distributed either randomly or uniformly in Bt and non-Bt corn ears for all three planting patterns and across all three trials (Table 5.4).

5.3.3 Occurrence and Distribution of Corn Earworm Larvae in Three Planting Patterns of Non-Bt and Bt Corn Containing Viptera 3111 Traits

The overall results of larval occurrence of corn earworm for a hybrid/planting pattern were also consistent across the four trials in which larval occurrence was investigated (Fig. 5.3).   The effect of treatment on larval occurrence was significant for all four trials ($F \geq$ 19.44; df = 3, 18; $P < 0.0001$) as well as for the pooled data ($F = 523.18$; df = 3, 81; $P < 0.0001$).   Across all four trials, an average of 3.00 larvae per plant was found on the ears of refuge plants in RIB plantings, which was significantly ($P < 0.05$) greater than that (2.35 /ear) observed in the pure stands of non-Bt plants (Fig. 5.3).   Viptera 3111 Bt corn plants were very effective for controlling corn earworm.   No live larvae were observed in the pure stands of Bt plants and only a total of five second instars (or an average of 0.01 larvae per plant) was recorded from Bt plants in RIB plantings (Fig. 5.3).

Larval distribution in pure stand of Bt corn plantings could not be analyzed because no live larvae were recorded from Bt corn ears in the four trials.   In pure stand of non-Bt corn plantings, like the egg distributions described above, larvae of corn earworm were also distributed either randomly or uniformly across the four trails (Table 5.4).   However, larvae of corn earworm were distributed aggregately in RIB plantings across all four trials, in which majority (92%) of the observed larvae inhabited on the refuge ears.
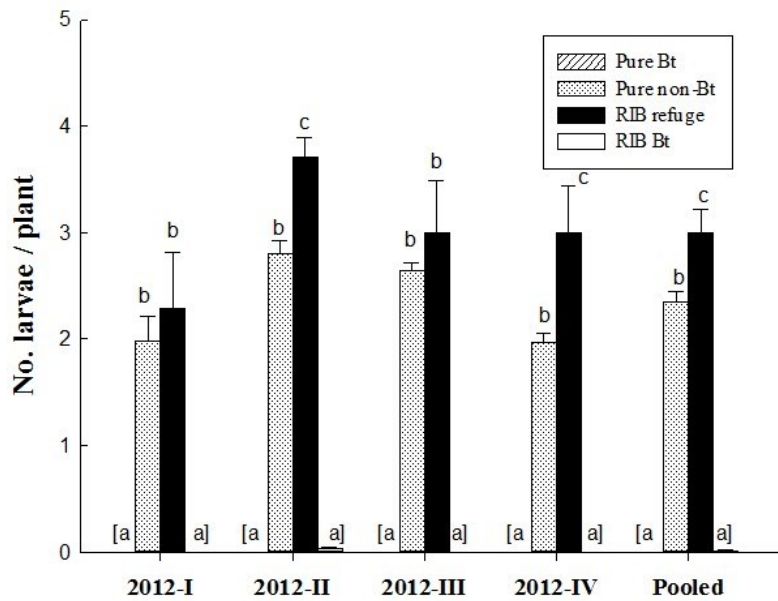
Fig. 5.3. Larval occurrence (mean ± sem) of *Helicoverpa zea* in three planting patterns of non-Bt and Bt plants containing Agrisure® Viptera™ 3111 traits. Means in each trial and combined analysis followed by the same letter were not significantly different (Tukey's HSD test, α = 0.05).
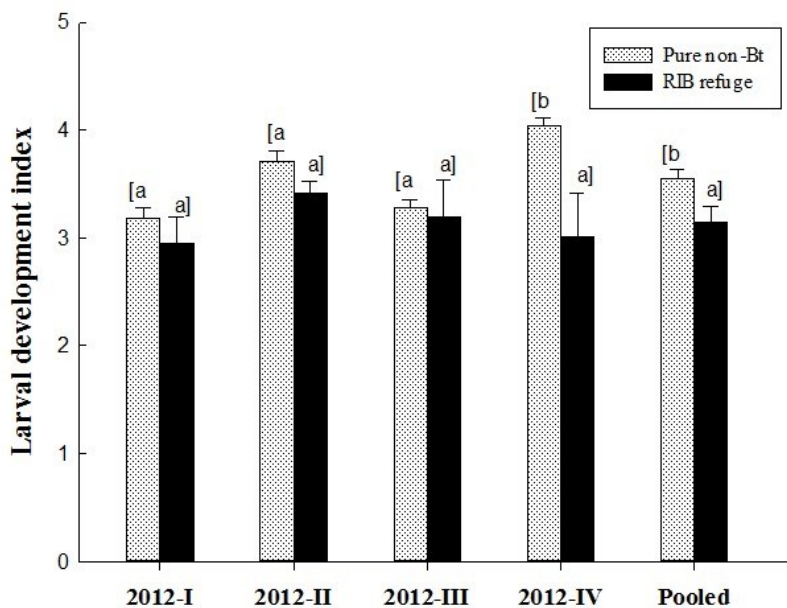
Fig. 5.4. Larval development index (mean ± sem) of *Helicoverpa zea* in three planting patterns of non-Bt and Bt plants containing Agrisure® Viptera™ 3111 traits. Means in each trial and combined analysis followed by the same letter were not significantly different (Tukey's HSD test, α = 0.05).

94

Table 5.3. Ear damage (mean $cm^2 \pm$ sem) of *Helicoverpa zea* in three planting patterns of non-Bt and Bt plants containing Agrisure[®] Viptera[TM] 3111 traits.

Planting pattern		2011-I	2011-II	2011-III	2011-IV	2012-I	2012-II	2012-III	2012-IV	Pooled
Pure Bt		0.00 ± 0.00 a	0.23 ± 0.12 a	0.00 ± 0.00 a	0.00 ± 0.00a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.02 ± 0.01 a
Pure non-Bt		1.50 ± 0.12 b	4.88 ± 0.27 b	3.38 ± 0.31 b	3.13 ± 0.43 b	5.76 ± 0.93 b	7.48 ± 0.69 b	5.45 ± 0.28 b	6.60 ± 0.78 b	4.96 ± 0.32 c
RIB	Non-Bt	0.00 ± 0.00 a	1.60 ± 1.03 a	6.71 ± 2.49 b	2.20 ± 0.20 b	4.21 ± 1.05 b	6.21 ± 0.90 b	5.43 ± 1.17 b	5.43 ± 1.27 b	4.30 ± 0.54 b
	Bt	0.00 ± 0.00 a	0.05 ± 0.03 a	0.04 ± 0.04 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.01 ± 0.01 a	0.00 ± 0.00 a	0.00± 0.00 a	0.01± 0.01 a
F-test	F-value	$F_{3,12} = 351.09$	$F_{3,12} = 14.83$	$F_{3,18} = 25.24$	$F_{3,12} = 161.52$	$F_{3,18} = 19.78$	$F_{3,18} = 172.14$	$F_{3,18} = 78.65$	$F_{3,18} = 65.02$	$F_{3,147} = 82.15$
	P-value	<0.0001	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[*]Means in a column followed by a different letter were significantly different (Tukey's HSD test, α=0.05).

Table 5.4. Egg and larval distribution of *Helicoverpa zea* in three planting patterns of non-Bt and Bt plants containing Agrisure® Viptera™ 3111 traits.

Trial	Plant pattern	Eggs		Larvae	
		Dispersion index (s^2/m)	Distribution	Dispersion index (s^2/m)	Distribution
2012-I	Pure Bt	1.302	Random	----	----
	Pure non-Bt	0.832	Random	0.916	Random
	RIB	0.646	Uniform	2.790	Aggregated
2012-II	Pure Bt	1.197	Random	----	----
	Pure non-Bt	1.250	Random	0.630	Uniform
	RIB	1.178	Random	3.373	Aggregated
2012-III	Pure Bt	0.752	Random	----	----
	Pure non-Bt	0.427	Uniform	0.720	Uniform
	RIB	0.863	Random	3.350	Aggregated
2012-IV	Pure Bt	----	----	----	----
	Pure non-Bt	----	----	0.581	Uniform
	RIB	----	----	3.270	Aggregated

5.3.4 Larval Development of Corn Earworm in Three Planting Patterns of Non-Bt and Bt Corn Containing Viptera 3111 Traits

Because no live larvae of corn earworm were observed from ears of pure stand of Bt corn plantings and few 2nd instar larvae were found from Bt corn ears of RIB plantings, statistical analyses were conducted only for the data collected from non-Bt plants. Therefore, treatment comparisons in larval development could be made only between ears of pure stand of non-Bt corn plantings and refuge ears of RIB plantings.   As shown in Fig. 5.4, there is a consistent trend that the development index of larvae recovered from refuge ears in

96

RIB plantings was numerically less than that of larvae found on ears of pure stand of non-Bt corn plantings.   The differences were significant (p < 0.05) for the trial 2012-IV as well as for the pooled data.   Across the four trials, average development index of larvae recovered from pure stands of non-Bt plants reached 3.55, compared to 3.14 for the larvae found on refuge ears in RIB plantings (Fig. 5.4).

5.3.5 Ear Damage by Corn Earworm in Three Planting Patterns of Non-Bt and Bt Corn Containing Viptera 3111 Traits

Effect of corn hybrid/planting pattern on ear damage area by corn earworm was significant for all eight trials ($F \geq 14.83$; df = 3, 12-18; $P \leq 0.0002$) as well as for the pooled data ($F = 82.15$; df = 3,147; $P < 0.0001$).   Except for the trials 2011-I and 2011-II, the overall results were largely consistent across the other six trials (Table 5.3).   According to the pooled data analysis, an average of 4.96 cm^2 /ear was damaged by corn earworm in pure stands of non-Bt plants, which was not significantly different ($P > 0.05$) from that observed on refuge plants in RIB plantings (4.30 cm^2/ear).   Across all eight trials, little or no damage was observed on ears of Bt plants in both pure stand of Bt corn and RIB plantings (Table 5.3).

5.4 Discussion

ELISA tests in this study showed that 100% of the F_2 corn kernels removed from Bt corn ears in pure Bt corn plantings expressed both Vip3A and Cry1Ab in both trials.   This suggests that the alleles of Vip3A and Cry1Ab are likely homozygous in the two parents of the F_1 corn hybrid.   Similarly, another independent study also reports that 100% F_2 kernels of a pure-planted Bt corn hybrid containing Genuity$^{®}$ SmartStax^{TM} traits expresses all Cry proteins that are presented in the F_1 plants (Yang et al., 2014b).   The results are controversial to a commonly assumption that 25% F_2 kernels should be homozygous for a particular Bt

gene in a corn hybrid, and 50% should be heterozygous for the Bt allele, while the other 25%

should not express the Bt protein (Chilcutt and Tabashink, 2004; Chilcutt et al., 2007;

Burkness et al., 2010; Burkness et al., 2011).   In addition, because corn is a cross-pollinating

crop with separate male and female flowering structures, in which most pollination results

from pollens dispersed by wind and gravity (Brittan, 2006), the kernels of F_2 refuge ears in

RIB plantings will likely be contaminated to express some levels of Bt proteins (Chilcutt and

Tabashnik, 2004; Burkness and Hutchison, 2012).   In the current study, we qualitatively

demonstrated that a great percentage (61.6%) of refuge kernels in RIB plantings was

contaminated to express Vip 3A or Cry1Ab protein due to the cross-pollination.   Limited by

the technology, concentration of each Bt protein in individual kernels was not measured in

this study.   However, as reported in Yang et al. (2014b), the clear and strong bands exhibited

in the ELISA test strips (Fig. 5.1) indicated that protein expression levels in the contaminated

kernels of refuge ears were not low.   Moreover, as observed for the Cry1A/Cry2A, Cry1F,

Cry3B, and Cry34/35A in Genuity® SmartStax™ corn (Yang et al., 2014b), χ^2-tests suggest

that the two Bt genes, Vip3A and Cry1Ab, also segregated independently in Viptera 3111

corn.   The verification of Bt protein expression in F_2 kernels and the demonstration of

independent segregation of Bt genes should provide useful information to improve modeling

IRM for Bt corn.

Compared to single-gene Bt corn, it is expected that pyramided Bt corn hybrids are more

effective against noctuid target species such as corn earworm and fall armyworm, *Spodoptera

frugiperda* (J.E. Smith) (Moar and Anilkumar, 2007; Monsanto, 2012; Syngenta, 2012).

However, there is little published data demonstrating the field efficacy of pyramided Bt corn

against these species, especially under RIB plantings.   Results of the multiple field trials in

the current study consistently demonstrated that Viptera 3111 corn products are highly

effective against field populations of corn earworm.   Corn plants containing Viptera 3111

traits offered virtually complete control in both pure stand and RIB plantings in the field trials

of this study.   Additionally, a previous five-year field study showed that Bt sweet corn

products containing the Viptera trait in pure stand plantings were also highly effective for

managing corn earworm (Burkness et al., 2010).   Furthermore, laboratory, greenhouse, and

limited field studies have suggested that Viptera Bt corn is extremely effective against fall

armyworm (Burkness et al., 2010; Yang et al., 2013; Niu et al., 2013, 2014).   Yang et al.

(2013) showed that in an F_2 screen on Viptera 3111 corn leaf tissue, all 14,400 F_2 neonates of

150 singe-pair families of fall armyworm were killed within 7 days.   Similarly, Niu et al.

(2013; 2014) demonstrated that larvae of a highly Cry1F-resistant population of fall

armyworm couldn't survive on either leaf tissue or whole plants of Viptera 3111 corn.

Although all of these studies were not particularly designed to evaluate the high dose

qualification as required by the US-EPA FIFRA Scientific Advisory Panel (US-EPA, 2001),

results of the current study, together with others, strongly suggest that Bt corn hybrids

containing Viptera trait (Vip3A+Cry1Ab) likely produce a "high-dose" against both corn

earworm and fall armyworm.   These two noctuid species are two important targets of the

second generation pyramided Bt corn in both North and South America (Burkness et al., 2011;

Niu et al., 2013; 2014).

Knowledge of oviposition behavior and larval movement of target pests is also useful

for developing effective IRM strategies for Bt crops (Davis and Onstad, 2000; Mallet and

Porter, 1992; Goldstein et al., 2010; Burkness et al., 2011; Ives et al., 2011; Onstad et al., 2011; Razz and Mason, 2012; Wangila et al., 2013).   For example, if a target species prefers to laying more eggs on non-Bt corn than Bt corn plants, and if there is only limited movement of the insect, a RIB planting could be more effective in providing refuge populations compared to structured refuge plantings.   However, if these same susceptible larvae disperse from non-Bt refuge plants to Bt plants in a RIB planting, it would likely result in greater mortality to susceptible populations than in structured refuge plantings and thus result in a lower refuge population (Davis and Onstad, 2000).   Results of this study showed that there were no significant differences in corn earworm egg occurrences between Bt and non-Bt corn ears and the eggs distributed either randomly or uniformly in all three planting patterns across all trials in which egg occurrences were investigated.   These results provide further evidence that corn earworm has no ovipositioning preference between Bt and non-Bt plants (Yang et al., 2014a).   Similarly, indiscriminate oviposition behavior between Bt and non-Bt plants has also been shown to occur in several other target species of Bt crops; including European corn borer, *Ostrinia nubilalis* (Hübner), pink bollworm, *Pectinophora gossypiella*, and cotton bollworm, *Helicoverpa armigera* (Orr and Landis, 1997; Hellmich et al., 1999; Hutchison et al., 2010; Liu et al., 2002; Dhillon and Sharma, 2013).   Thus, it appears that indiscriminate oviposition between Bt and non-Bt plants is a ubiquitous behavior among many insect species.   In this study, as similarly reported in Yang et al. (2014a), larvae of corn earworm, in pure stands of non-Bt corn, exhibited the same distribution patterns (uniformly or randomly) that were observed for the egg distributions.   The results again suggest that larval movement of corn earworm among plants is likely to be limited in pure stand of non-Bt corn

plantings.   Also as reported in Yang et al. (2014a), due to the high toxicity of Viptera 3111 plants to corn earworm, almost all live larvae collected in the RIB plantings were from refuge ears, resulting in an aggregated field distribution as shown in Table 5.2.   The similar results observed between the current and the previous studies (Yang et al., 2014a) with Genuity® SmartStax™ suggest that the oviposition behavior and larval distribution of corn earworm is likely independent of corn hybrids or Bt corn traits.

Similar to the report in Yang et al. (2014a), data of this study suggested that the Bt protein contamination in RIB plantings did not significantly reduce larval populations of corn earworm at the early larval stages (e.g. $\leq 4^{th}$ instar) but significantly delayed larval development.   We understand that there were some limitations in both the current and previous (Yang et al., 2014a) field trials in determination of the suitability of RIB plantings for IRM.   As mentioned in Yang et al. (2014a), the experimental designs evaluated the effect on only the early larval stages of corn earworm because larger larvae like to cannibalize and drop into soil for pupation.   However, information generated from these studies should provide a good foundation for further studies to determine the effect of the protein contamination in RIB plantings on the entire life cycle of corn earworm.   In addition, as described above, the results of this study were consistent with the previous research with Genuity® SmartStax™ (Yang et al., 2014a), suggesting that the biological parameters of corn earworm generated from these studies might also be applied for other Bt corn traits in IRM modeling.

5.5 References

Brittan, K., 2006. Methods to enable the coexistence of diverse corn production system. Agri. Biotechnol. Cal. Ser. Publication 88192. Available at: http://anrcatalog.ucdavis.edu/pdf/8192.pdf.

Burkness, E.C., Dively, G., Patton, T., Morey, A.C., Hutchison, W.D., 2010. Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae) under field conditions. GM crops 1, 1-7.

Burkness, E.C., Hutchison, W.D., 2012. Bt pollen dispersal and Bt kernel mosaics: integrity of non-Bt refugia for lepidopteran resistance management in maize. J. Econ. Entomol. 105, 1477-1870.

Burkness, E.C., O'Rourke, P.K., Hutchison, W.D., 2011. Cross-pollination of nontransgenic corn ears with transgenic Bt corn: efficacy against lepidopteran pests and implications for resistance management. J. Econ. Entomol. 104, 1476-1479.

Chilcutt, C.F., Odvody, G.N., Correa, J.C., Remmers, J., 2007. Effects of *Bacillus thuringiensis* transgenic corn on corn earworm and fall armyworm (Lepidoptera: Noctuidae) densities. J. Econ. Entomol. 100, 327-334.

Chilcutt, C.F., Tabashnik, B.E., 2004. Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maize. Proc. Natl. Acad. Sci. USA 101, 7526-7529.

Davis, P.M., Onstad, D.W., 2000. Seed mixtures as a resistance management strategy for European corn borers (Lepidoptera: Crambidae) infesting transgenic corn expressing Cry1Ab protein. J. Econ. Entomol. 93, 937-948.

Dhillon, M.K., Sharma, H.C., 2013. Comparative studies on the effects of Bt-transgenic and nontransgenic cotton on arthropod diversity, seedcotton yield and bollworms control. J. Environ. Biol. 34, 67-73.

Dhurua, S., Gujar, G.T., 2011. Field-evolved resistance to Bt protein Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) from India. Pest Manag. Sci. 67, 898-903.

Difonzo, C., Collen, E., 2012. Handy Bt trait table. Available at: http://www3.ag.purdue.edu/agry/PCPP/Documents/Entry%20forms/Handy_Bt_Trait_Table.pdf.

Gassmann, A.J., Petzold-Maxwell, J.L., Keweshan, R.S., Dunbar, M.W., 2011. Field-evolved resistance to Bt maize by western corn rootworm. PloS ONE 6, e22629.

Goldstein, J.A., Mason, C.E., Pesek, J., 2010. Dispersal and movement behavior of neonate European corn borer (Lepidoptera: Crambidae) on non-Bt and transgenic Bt corn. J. Econ. Entomol. 103, 331-339.

Hellmich, R.L., Higgins, L.S., Witkowski, J.F., Campbell, J.E., Lewis, L.C., 1999. Oviposition by European corn borer (Lepidoptera: Crambidae) in response to various transgenic corn events. J. Econ. Entomol. 92, 1014-1020.

Huang, F., Andow, D.A., Buschman, L.L., 2011. Success of the high dose/refuge resistance management strategy after 15 years of Bt crop use in North America. Entom. Exp. App. 140, 1-16.

Hutchison, W.D., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S.J., Abrahamson, M., Hamilton, K.L., Steffey, K.L., Gray, M.E., et al., 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt mazie growers. Science, 222-225.

Ives, A.R., Glaum, P.R., Ziebarth, N.L., Andow, D.A., 2011. The evolution of resistance to two-toxin pyramided transgenic crops. Ecol. App. 21, 503-515.

James, C., 2013. Global status of commercialized biotech/GM crops: 2013. *ISAAA Brief* No. 46. ISAAA: Ithaca, NY, USA.

Kurtz, R.W., 2010. A review of Vip3A mode of action and effects on Bt Cry protein-resistant colonies of lepidopteran larvae. Southwest. Entomol. 35, 391-394.

Liu, Y.B., Tabashnik, B.E., Dennehy, T.J., Carrière, Y., Sims, M.A., Meyer, S.K., 2002. Oviposition on and mining in bolls of Bt and non-Bt cotton by resistant and susceptible pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 95, 143-148.

Mallet, J., Porter, P., 1992. Preventing insect adaptation to insect-resistant crops are seed mixtures or refugia the best strategy? Proc. R. Soc. Lond. B. Biol. Sci. 250,165-169.

Matten, S.R., Frederick, R.J., Reynolds, A.H., 2012. United States Environmental Protection Agency insect resistance management programs for plant-incorporated protectants and use of simulation modeling. pp 175-267. *In:* C.A. Wozniak, A. McHughen (eds), "Regulation of agricultural biotechnology: The United States and Canada". Springer.

Matten, S.R., Head, G.P., Quemada, H.D., 2008. How governmental regulation can help or hinder the integration of Bt crops into IPM programs. *Integration of Insect-Resistant Genetically Modified Crops within IPM Programs*, eds Romeis J, Shelton AM, Kennedy GG (Springer, New York), pp. 27-39.

Moar, W.J., Anilkumar, K.J., 2007. The power of the pyramid. Science 318, 1561-1562.

Monsanto. 2012. IRM grower guide: insect resistance management for U.S. corn and cotton-growing areas. Available at: http://www.monsanto.com/products/Pages/insect-resistance-management.aspx.

Niu, Y., Meagher-Jr, R., Yang, F., Huang, F., 2013. Susceptibility of field populations of the fall armyworm (Lepidoptera: Noctuidae) from Florida and Puerto Rico to purified Cry1F protein and corn leaf tissue containing single and pyramided Bt genes. Fla. Entomol. 96, 701-713.

Niu, Y., Yang, F., Dangal, V., Huang, F., 2014. Larval survival and plant injury of Cry1F-susceptible, -resistant, and -heterozygous fall armyworm (Lepidoptera: Noctuidae) on non-Bt and Bt corn containing single or pyramided genes. Crop Prot. 59, 22-28.

Onstad, D.W., Mitchell, P.D., Hurley, T.M., Lundgren, J.G., Porte, R.P., Krupke, C.H., Spencer, J.L., DiFonzo, C.D., Baute, T.S., Hellmich, R.L., Buschman, .LL., Hutchison, W.D., Tooker, J.F., 2011. Seeds of change: corn seed mixtures for resistance management and IPM. J. Econ. Entomol. 104, 343-352.

Orr, D.B., Landis, D.A., 1997. Oviposition of European corn borer (Lepidoptera: Pyralidae) and impact of natural enemy populations in transgenic versus isogenic corn. J. Econ. Entomol. 90, 905-909.

Ostlie, K.R., Hutchison, W.D., Hellmich, R.L., 1997. Bt-Corn & European Corn Borer, Long Term Success through Resistance Management. North Central Region Extension Publication 602. University of Minnesota, St. Paul, MN, USA.

Razze, J.M., Mason, C.E., 2012. Dispersal behavior of neonate European corn borer (Lepidoptera: Noctuidae) on Bt corn. J. Econ. Entomol. 105, 1214-1223.

Reynolds, A., 2008. Review of an Amendment Request to Reduce the Refuge Required for MON 89034 in the Corn Belt. Memorandum to Jeannine Kausch, Regulatory Action Leader, Microbial Pesticides Branch, Biopesticides and Pollution Prevention Division. (Environmental Protection Agency, Washington, DC).

Roush, R.T., 1998. Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? Phil. Trans. R. Soc. Lond. B 353, 1777-1786.

SAS Institute. 2010. SAS/ STAT User's Third Edition, SAS Institute Inc, Cary, NC.

Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W., Huckaba, R.M., 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 103, 1031-1038.

Syngenta. 2012. Corn trait Information: Agrisure Viptera™ 3111. Available at: http://www.syngenta.com/country/us/en/Seeds/Traits/CornTraits/Pages/content_authorin g_Agrisurevip3111.aspx.

USDA-NASS (National Agricultural Statistics Service). 2013. Acreage. USDA, Washington DC. USA. Available at: http://usda01.library.cornell.edu/usda/current/Acre/Acre-06-28-2013.pdf.

US-EPA (United States Environmental Protection Agency). 2001. Biopesticide registration action document: *Bacillus thuringiensis* plant-incorporated protectants. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm.

US-EPA (United States Environmental Protection Agency). 2009. Biopesticide registration action document: *Bacillus thuringiensis* Vip3Aa20 insecticidal protein and the genetic material necessary for its production (*via* elements of vector pNOV1300) in event MIR162 maize (OECD unique identifier: SYN-IR162-4). Available at: http://www.epa.gov/opp00001/biopesticides/ingredients/tech_docs/brad_006599.pdf.

US-EPA (United States Environmental Protection Agency). 2010. Biopesticide registration action document: *Bacillus thuringiensis* Cry1Ab and Cry1F *Bacillus thuringiensis* (Bt) corn plant-incorporated protectants. Available at: http://www.epa.gov/pesticides/biopesticides/pips/cry1f-cry1ab-brad.pdf

Van-Rensburg, J.B.J., 2007. First report of field resistance by stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. S. Afr. J. Plant Soil. 24, 147-151.

Wangila, D.S., Leonard, B.R., Bai, Y., Head, G.P., Huang, F., 2012. Larval survival and plant injury of Cry1Ab-susceptible, -resistant, and -heterozygous genotypes of the sugarcane borer on transgenic corn containing single or pyramided Bt genes. Crop Protect. 42, 108-115.

Wangila, D.S., Leonard, B.R., Ghimire, M.N., Bai, Y., Zhang, L., Yang, Y., Emfinger, K.D., Head, G.P., Yang, F., Niu, Y., Huang, F., 2013. Occurrence and larval movement of *Diatraea saccharalis* (Lepidoptera: Crambidae) in seed mixes of non-Bt and Bt pyramid corn. Pest Manag. Sci. 69,1163-1172.

Yang, F., Huang, F., Qureshi, J.A., Leonard, B.R., Niu, Y., Zhang, L., Wangila, D.S., 2013. Susceptibility of Louisiana and Florida populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to transgenic Agrisure [®] Viptera[TM] 3111 corn. Crop Protect. 50, 37-39.

Yang, F., Kerns, D.L., Head, G.P., Leonard, B.R., Levy, R., Niu, Y., Huang, F., 2014b. A challenge for the seed mixture refuge strategy in Bt maize: impact of cross-pollination on an ear-feeding pest, corn earworm. PLoS ONE (In press).

Yang, F., Kerns, D.L., Head, G.P., Leonard, B.R., Niu, Y., Huang, F., 2014a. Occurrence, distribution, and ear damage of *Helicoverpa zea* (Lepidoptera: Noctuidae) in mixed plantings of non-Bt and Bt corn containing Genuity® SmartStax™ traits. Crop Protect. 55, 127-132.

Zar, J.H., 1984. Biostatistical analysis, 2nd ed. Prentice-Hall, Englewood Cliffs, NJ.

Zhao, J.Z., Cao, J., Li, Y., Collins, H.L., Roush, R.T., Earle, E.D., Shelton, A.M., 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat. Biotechnol. 21, 1493-1497.

CHAPTER 6. A CHALLENGE FOR THE SEED MIXTURE REFUGE STRATEGY IN BT CORN: IMPACT OF CROSS-POLLINATION ON AN EAR-FEEDING PEST, CORN EARWORM[5]

6.1 Introduction

Transgenic crops (corn and cotton) expressing *Bacillus thuringiensis* (Bt) proteins were planted on >178 million acres for pest control in the world in 2013 (James, 2013). In the U.S., 76% of the field corn was planted to Bt corn in the same year (NASS, 2013). Field performance of Bt crops, in general, has been very effective against the target insect pests (Hutchison et al., 2010; Edgerton et al., 2012; Siebert et al., 2012; Yang et al., 2014). However, the intensive use of Bt crops places high selection pressure on the target pest populations that could lead to the rapid evolution of resistance (Gould, 1998; Van-Rensburg, 2007; Store et al., 2010; Dhurua and Gujar, 2011; Gassmann et al., 2011; Huang et al., 2011; Tabashnik et al., 2013). To delay resistance development, a 'high dose/refuge' strategy has been adopted for planting Bt corn in the U.S. and several other countries (Ostlie et al., 1997; Gould, 1998; US-EPA, 2001; Matten et al., 2012). Before 2010, the refuge was required to be arranged in a structured form that was implemented as blocks or strips of non-Bt crops to maintain susceptible insect populations (Ostlie et al., 1997; US-EPA, 2001). Concerns with low compliance in the structured forms led to the introduction of a seed mixture strategy, also called RIB (refuge-in-the-bag), as an alternative approach for implementing refuge for Bt corn products that contains two or more pyramided Bt grenes for a target pest (Matten et al., 2012). Since 2010, a RIB approach of 95: 5% (Bt: non-Bt corn seeds) has been approved in the U.S, and adopted by growers, for several pyramided Bt corn products in the U.S. Corn

---

[5] In Press for publication in PLOS ONE

Belt (Mallet and Porter, 1992; Onstad et al., 2011; US-EPA, 2010a; Matten et al., 2012).

Pyramided Bt corn hybrids contain two or more Bt genes targeting the same pest species

(Roush, 1998; Zhao et al., 2003; Difonzo and Collen, 2012).   Due to differences in the

predominant pests from the Corn Belt, and particularly the presence of the ear-feeding pest

corn earworm, *Helicoverpa zea* (Boddie) as an overwintering insect (US-EPA, 2010a; 2010b ;

Alyokhin, 2011; Onstad et al., 2011; Kang et al., 2012), a RIB strategy has not been approved

in the southern U.S (Matten et al., 2012; Monsanto, 2012).   A major concern in

implementing RIB is cross-pollination of corn hybrids that can cause Bt proteins to be present

in refuge corn kernels in seed mix plantings (Chilcutt and Tabashnik, 2004; Burkness et al.,

2011; Burkness and Hutchison, 2012).   The Bt protein contamination in RIB could

negatively affect (e.g., survival, growth, and development) refuge insects, if they are ear

feeders.   However, prior to this study, the intensity of Bt protein contamination in RIB and its

associated effects on refuge populations of ear feeders under real field conditions have not been

investigated.   Argument over the effectiveness of "RIB" strategies for resistance management

has been a hot topic for two decades and heated debates continue among growers, seed

companies, extension specialists, research scientists, and regulators (Mallet and Porter, 1992;

Davis and Onstad, 2000; Chilcutt and Tabashnik, 2004; Burkness and Hutchison, 2012;

Carroll et al., 2012; 2013).

Corn earworm is a major target species of pyramided Bt corn in both North and South

America and its damage to corn is primarily caused by larvae feeding on ear kernels (Lindgren

et al., 1994).   Thus, the RIB-corn earworm system provides an excellent model to study the

effect of cross-pollination on refuge populations of ear feeding species.   As mentioned

above, the effect of Bt protein contamination in refuge kernels due to pollen movement on refuge populations of corn earworm couldn't be measured accurately if the analysis was relied on only the data obtained from the field observations as described in the chapters 4 and 5.   Because majority field populations of corn earworm feed on corn ears and mature larvae move out from the corn ears or plants and then drop into the soil for pupation (Capinera, 2000).   It is very difficult, if not impossible, to measure the impact of such effect on the entire life cycle of this species in open field studies.   The objective of this chapter is particuly designed to address this limitations in the chapters 4 and 5.   To achieve this goal, three field, four laboratory and three field-plus-laboratory studies were conducted in 2012-2013 to assess the intensity of Bt protein contamination in RIB plantings of non-Bt and Bt corn containing Genuity® SmartStax™ trait and the corresponding effect on survival, growth, and development of corn earworm.   Genuity® SmartStax™ is a common pyramided Bt corn product, which expresses Cry1A.105, Cry2Ab2 and Cry1F targeting lepidopteran pests and Cry3Bb1, Cry34/35Ab1 for controlling underground coleopteran rootworms (Monsanto, 2012).   The results show that the RIB approach is not effective for providing refuge for corn earworm.   Our study is timely given growing concerns over the resistance management for Bt crops.   It is also an important guide for regulators in making science-based decisions regarding the suitability of seed mixture strategies for different regions.

6.2 Materials and Methods

6.2.1 Sources of Corn and Insects

Three Bt corn hybrids (DKC 61-21, DKC 55-09, and DKC 62-08, Monsanto, St. Louis MO) containing the Genuity® SmartStax™ trait and two closely related non-Bt corn hybrids

(DKC 61-22 and DKC 66-49, Monsanto, St. Louis MO) were used in this study.   Genuity[®]

SmartStax[TM] contains six Bt genes including three genes, Cry1A.105, Cry2Ab2, and Cry1F,

for controlling above-ground lepidopteran species, and three genes, Cry3Bb1, Cry34Ab1, and

Cry35Ab1 for managing under-ground rootworms, *Diabrotica* spp. (Difonzo and Collen,

2012).

Laboratory populations of corn earworm were established from feral larvae (~100

individuals for each population) collected from non-Bt corn fields in Rapides Parish,

Louisiana and Hidalgo County, Texas, USA.   Field collected larvae were individually reared

in 30-ml plastic cups containing a pre-mixed meridic diet (WARD'S Stonefly Heliothis diet,

Rochester, NY).   Pupae removed from the rearing cups were placed into ~20 L mesh cages

(Seville Classics, INC., Torrance, CA) containing ~200g vermiculite (Sun Gro, Pine Bluff,

AR) and 10% honey water solution.   The cages were then placed in growth chambers at

26.8°C, >90% RH and a 14:10 h (L: D) photoperiod for adult emergence, mating, and

oviposition. F_1 neonates (<24 h old) from the field-collected corn earworm were used,

except where otherwise specified, in all field trials and laboratory bioassays in this study.

6.2.2 Field Planting

A total of three field trials were conducted in two locations in Louisiana, USA in 2012

(one trial) and 2013 (two trials).   The 1st (2012) and 2nd (2013) trials were located in

Franklin Parish (32°08'N; 91°41'W) in northeast Louisiana and the 3rd trial was conducted in

Rapides Parish (31°10'35.99"N; 92°23'24.24"W) in central Louisiana in 2013.   Each trial

consisted of three planting patterns: 1) pure stand of Bt plants, 2) pure stand of non-Bt plants,

and 3) RIB planting of 95% Bt and 5% non-Bt (refuge) plants.   There was a distance

of >300 m between fields, and no other corn plants at similar growth stages were planted

within 300 m of the trial fields.   The designed isolation should avoid any cross-pollination

among corn fields (Bannert, 2006).

In each trial, there were ~ 4000-6000 plants in 12 rows (centered on 102 cm between

two rows and 15.2 cm between two seeds in a row) in each of two pure standing planting

fields and 5400 plants for each RIB planting which included 270 non-Bt refuge plants and

5130 Bt plants.   The non-Bt corn seeds were manually planted in a uniform pattern across

the RIB fields right after the Bt corn seeds were planted.   At each location, two non-Bt seeds

were planted ~5 cm apart and ~2.5 cm off center of the row of Bt corn seeds and marked with

wooden stakes.   After about two weeks, refuge plants were thinned to one plant for each

spot and tagged with colored vinyl tape.   At the same time, the Bt plant that was closest to

the non-Bt plant was removed to maintain the designed plant density and spacing.   Irrigation,

fertilization, and other management practices were used as needed to ensure optimum growth

of the corn plants.   Presence/absence of the Bt proteins were confirmed by testing leaf

samples from each planting pattern with QuickStix^{TM} Combo ELISA Kit (EnviroLogix, ME,

USA).   Primary ears of the three field trials were used in analyzing Bt protein expression

and assessing effect of Bt protein contamination in refuge ears on survival, growth, and

development of corn earworm with three methods: in-field observation, lab assay, and

field-plus-lab assay.

6.2.3 Analysis of Protein Expression

In each trial, primary ears of 13 non-Bt corn refuge plants were randomly sampled from

RIB plantings during R2-R3 stages.   At the same time, 10 Bt and 10 non-Bt corn ears were

also randomly collected from the pure Bt and pure non-Bt fields, respectively. For ears that were sampled from RIB and pure-Bt plantings, five kernels from the top to bottom of each ear were removed and then individually examined for expression of Cry proteins using the QuickStix™ Combo kit (Fig. 6.1). For the ears collected from pure non-Bt corn field, 25 kernels were randomly sampled from each ear and pooled for analysis to validate the absence of Bt protein expression. Because the genes Cry1A.105 and Cry2Ab2 as well as Cry34Ab1 and Cry35Ab1 were linked in Genuity® SmartStax™, the ELISA Combo Kit identifies the six individually Bt proteins as four groups: Cry1A/Cry2Ab, Cry1F, Cry3Bb, and Cry34/35Ab1.
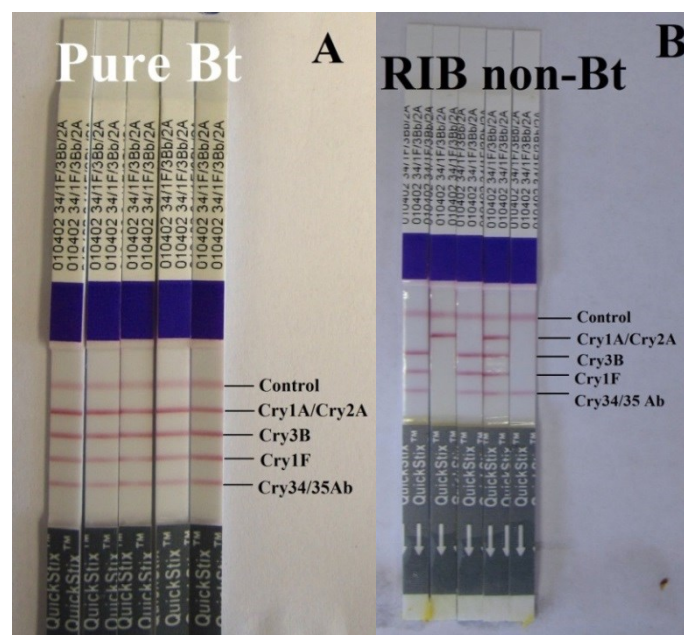
Fig. 6.1. Demonstration of Bt protein expression in individual kernels removed from ears of pure Genuity® SmartStax™ planting (A) and refuge ears of RIB (B) on QuickStix™ Combo ELISA test strips (EnviroLogix, ME, USA).

6.2.4 In-Field Observation

Survival, growth, and development of corn earworm on ears from the three planting patterns were first investigated using an in-field observation method with artificial insect

infestation.   To ensure sufficient pollinations, artificial infestations were conducted ~7 days

after the peak of pollination when plants were at R2 stage (Abendroth et al., 2011).   Each

in-field observation consisted of six treatments, one with ears from pure non-Bt plantings,

one with ears from pure Bt corn plantings, and four with ears from RIB plantings.   From

each RIB planting field, 40 refuge plants, and their primary ears, were first randomly selected

and then six primary ears from neighboring Bt plants at each sampled refuge plant were

selected as shown in Fig. 6.2.   To facilitate data presentation, the seven plants selected in

each location from RIB plantings were considered as four treatments: 1) RIB refuge: the

refuge plant; 2) A1-Bt: the Bt plants immediately adjacent to and within the same row as the

refuge plant (two plants total); 3) A3-Bt: the 3rd Bt plants on both sides of the refuge plant in

the same row (two plants total), and 4) B-Bt: the closest Bt plants on both sides of the refuge

plant in the two adjacent rows (two plants total).   At the same time, 20 plants were also

randomly selected in each of the pure Bt and pure non-Bt corn fields, respectively.   Before

artificial infestation, naturally occurring corn earworm larvae/eggs, if any, were removed

from the ears of the selected plants and then two neonates (<24 h old) were manually placed

on the top of each ear to simulate the natural field infestation.   After release of the neonates,

ears were covered with 17.8-cm corn ear shoot bags (Southern Exposure Seed Exchanges,

Mineral, VA, USA).   The open end of the ear shoot bags was attached tightly to the ear

surface so that any larval movement out of the bags would be apparent based on the exit

holes in the bags.

Larval survival and development were checked at 6-day after neonate release and every

3 days thereafter until larvae moved out of the ears or died (Hardwick, 1965).   Under field

conditions, mature larvae of corn earworm usually drop from the ears to pupate in the soil (Capinera, 2000), leaving boring holes in the shoot bags.   For data recording purposes, individuals that exited the bags in the later observations (e.g. at 12-day, 15-day and 18-day) were considered alive.   Therefore, survivorship of corn earworm for the in-field observation was calculated based on the total live larvae inside the bags and the number of exit holes in the shoot bags.   A complete block design was used for the in-field observation with trial as the block factor.   There were 20 to 40 ears (or 40 to 80 larvae) for each treatment replication.

6.2.5 Lab Assay

Because the in-field observation described above could not measure the impact of Bt contamination in refuge ears after mature larvae had exited from the ears and dropped into soil for pupation, a total of four lab assays were conducted using ears collected from the three field trials in 2012 and 2013.   Ears used in each lab assay were selected from the trial fields with the same sampling patterns (Fig. 6.2) as described in the in-field observation.   Selected ears along with husks and shanks were brought to the laboratory and naturally infested larvae, if any, were removed.   Ears sampled from the 1st field trial in 2012 were used in the first two lab assays (Lab assay-1 and Lab assay-2), while ears collected from each of the 2nd and 3rd field trials in 2013 were used in the 3rd (Lab-array-3) and 4th (Lab-array-4) lab assays, respectively.   Ears for the 1st, 3rd, and 4th lab assays were collected on the same days as the artificial infestations were performed for the field trials, while ears for the 2nd assay were sampled 5 days after the collections for the 1st lab assay.
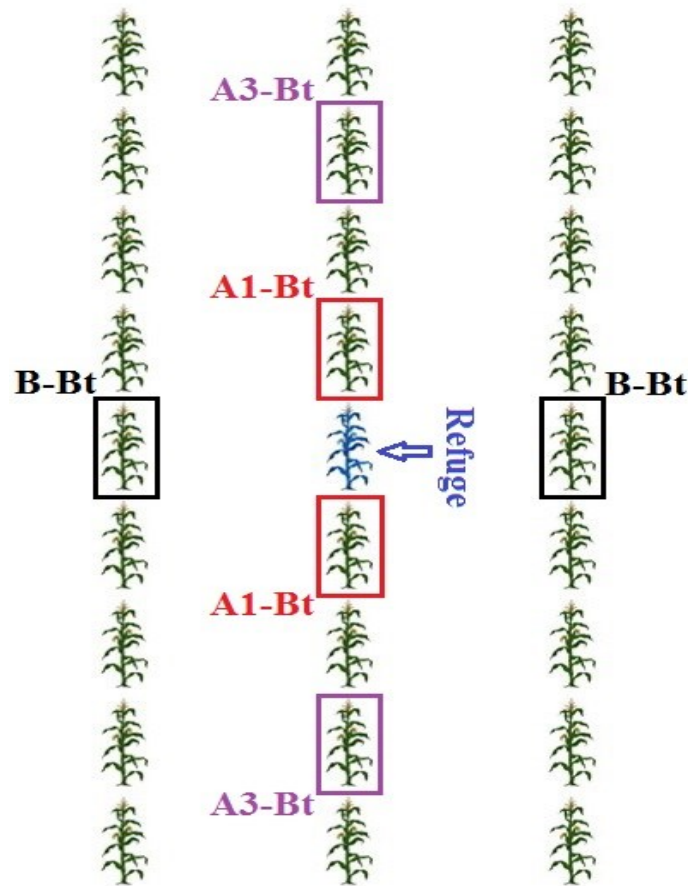
Fig. 6.2. A diagram showing the seven plants (four treatments) in each randomly selected location in a RIB planting that was used for the in-field observations and lab-bioassays. RIB refuge: the refuge plant; A1-Bt: the Bt plants immediately adjacent and within the same row as the refuge plant; A3-Bt: the 3rd Bt plants on both sides of the refuge plant in the same row; and B-Bt: the closest Bt plants on both sides of the refuge plant in the two adjacent rows.

In the lab assay, each ear was manually infested with two neonates on the top of each ear as described for the in-field observation. To maintain a suitable moisture level and keep the corn ear fresh during the test period, after insect infestation, shanks of the ears were inserted into water-satiated Jiffy-7® peat pellets (Jiffy Greenhouse, Fulton, KY, USA). Infested ears with the peat pellets attached were then placed into 5.7L plastic containers (one ear/container) (Sterilite Corporation, Townsend, MA, USA) with 2-3 pieces of paper towel underneath. The insect assay containers were placed into growth chambers maintained at 28°C, ~50% RH, 16L: 8D photoperiod. Survival, growth, and development of corn earworm were checked

115

after 6 days and every 3 days thereafter until adult emergence or death (Hardwick, 1965). A

complete block (growth chamber) design was used in each lab assay with 3 (Lab-array-1) or

4 (Lab-array-2, -3 and -4) replications and 8-10 ears/replication.

6.2.6 Field-Plus-Lab Assay

This method used field-collected ears containing naturally occurring early-stage corn

earworm larvae (3rd to 4th instars).   Previous field studies have shown that natural

occurrence of corn earworm on refuge ears in RIB plantings was not affected by Bt protein

contamination at the early larval stages (e.g. 3rd-4th instars) (Yang et al., 2014).   This result

led to the use of a field-plus-lab assay method to shorten the necessary laboratory assay

duration so that the effect, if any, of Bt protein degradation in detached ears on the results

could be minimized.   A total of three field-plus-lab assays were conducted using ears

collected from the two field trials in 2013; two assays using ears from the field trial in

Franklin Parish and another from the trial in Rapides Parish.   Because there were virtually

no live larvae on the ears of Bt corn plants when ear samplings were performed, each assay

consisted of only two treatments: 1) refuge ears from RIB and 2) ears of pure non-Bt corn

planting.   In each assay, ears with naturally occurring larvae along with husks and shanks

were sampled from field at the peak population of the 3rd instar stages and brought to the

laboratory.   The initial number of larvae and their corresponding developmental stages on

each ear were recorded while leaving larvae intact inside the ears.   The ears with the peat

pellets attached described above along with intact naturally occurring larvae were then placed

into plastic containers and maintained in the same conditions as described in the lab assay.

Survival, growth, and development of insects were checked every 2-3 days until adult

116

emergence or death (Hardwick, 1965).   A complete block design was used in the

field-plus-lab assay with assay as the block factor.   The number of ears used in each

treatment replication varied from 20 to 100 depending on the number of infested ears

available.

6.2.7 Data Analysis

Percent of kernels containing one or more Bt proteins was calculated based on the

number of kernels expressing the Bt proteins divided by the total kernels assayed.   Based on

presence/absence of the protein expression in kernels of the refuge ears, χ^2-tests were used to

analyze if the four gene groups in Genuity$^®$ SmartStax^{TM} segregated independently.   Then χ^2

value was determined using the equation: $\chi^2 = (n/100) [(O - E)^2/E + (E - O)^2/(100-E)]$.   Here,

n = number of kernels examined, O = observed percentage of kernels expressing the Bt

proteins, and E = expected percentage of kernels expressing the Bt proteins.   The E value for

a combination of two or more proteins was based on the assumption of independent

segregation.   For example, expected frequency of Cry1A/Cry2A + Cry3B was calculated

using the observed frequency of Cry1A/Cry2A timed by the observed frequency of Cry3B.

Insect developmental stages were converted to a development index: $1 = 1^{st}$ instar, $2 = 2^{nd}$

instar, …, $6 = 6^{th}$ instar, 7 = pupa as described in Yang et al. (2014).   Data on insect

survivorship, pupation, and moth emergence rate were transformed to arcsine square-root

value, while number of insect, development index, and pupal mass were converted to ln (x +

1) scale for normal distribution (Zar, 1984).   Transformed data were then analyzed using

one-way analysis of variance (ANOVA) (SAS Institute, 2010).   In addition, data for each

variable observed in lab assay were also pooled across the four assays and the pooled data were

117

analyzed using mixed models with assay as a random factor (SAS Institute, 2010).   For all

ANOVAs, treatment means were compared and separated by Tukey's HSD tests at $\alpha = 0.05$

level.   Untransformed data are presented in the tables and figures.

6.3 Results

6.3.1 Bt Protein Expression of Refuge Kernels in RIB Plantings

Qualitative ELISA tests (Fig. 6.1) showed that all 150 individual kernels sampled from

30 ears of pure Bt corn plantings in three field trials expressed all four Bt protein groups (six

Bt proteins) in Genuity® SmartStax™ (Table 6.1).   Similarly, all kernels from 30 ears from

pure non-Bt plantings were free of Bt protein expression, suggesting that there was no

cross-pollination among the trial fields.   However, cross-pollination within RIB fields

resulted in most (94.4%) refuge ear kernels expressing at least one Bt protein.   Frequency of

Bt protein expression in refuge kernels was consistent among the three field trials with an

average of 55.1, 29.5, 14.0, and 5.1% kernels expressing one to four groups of Bt proteins,

respectively (Table 6.1).   Based on the Bt expression recorded in refuge kernels, χ^2 tests

showed that the four protein groups segregated independently in all three trials with only a

few exceptions (Table 6.1).

6.3.2 In-Field Observation for Survival and Development of Corn Earworm

Genuity® SmartStax™ is very effective against corn earworm.   The survivalship of

corn earworm larvae on the Bt corn ears was significantly ($P < 0.0001$) less than that on the

RIB refuge and pure non-Bt ears during the test periods (Table 6.2).   Results of in-field

observation also showed no corn earworm neonates developed to the pupal stage on ears of

Bt plants either in pure Bt corn plantings or RIB (Fig. 6.3A).   Bt protein contamination in

Table 6.1. Percentage (mean ± sem) of individual kernels expressing Bt proteins in Genuity® SmartStax™ maize in pure Bt, pure non-Bt, and RIB plantings [a].

| Bt protein group | Field trial 1 | | | | | Field trial 2 | | | | | Field trial 3 | | | | | Pooled[c] | | | | |
| | Pure Bt (%) | Pure non-Bt (%) | RIB refuge | | | Pure Bt (%) | Pure non-Bt (%) | RIB refuge | | | Pure Bt (%) | Pure non-Bt (%) | RIB refuge | | | Pure Bt (%) | Pure non-Bt (%) | RIB refuge | | |
			O[b] (%)	E[b] (%)	χ^2			O (%)	E (%)	χ^2			O (%)	E (%)	χ^2			O (%)	E (%)	χ^2
Cry1A/Cry2A	100	0	47.7 ± 8.0	---	---	100	0	50.8 ± 7.0	---	---	100	0	49.2 ± 7.7	---	---	100	0	49.2 ± 4.3	---	---
Cry3B	100	0	53.9 ± 4.7	---	---	100	0	96.9 ± 3.1	---	---	100	0	72.3 ± 5.8	---	---	100	0	74.4 ± 3.9	---	---
Cry1F	100	0	56.9 ± 5.9	---	---	100	0	41.5 ± 5.3	---	---	100	0	36.9 ± 6.7	---	---	100	0	45.1 ± 3.7	---	---
Cry34/35Ab	100	0	46.2 ± 6.6	---	---	100	0	60.0 ± 4.5	---	---	100	0	49.2 ± 7.7	---	---	100	0	51.8 ± 3.7	---	---
Cry1A/Cry2A + Cry3B	100	0	26.2 ± 5.7	25.7	0.01	100	0	50.8 ± 7.0	49.2	0.06	100	0	36.9 ± 7.5	35.6	0.05	100	0	38.0 ± 4.1	36.6	0.15
Cry1A/Cry2A + Cry1F	100	0	26.2 ± 6.9	27.2	0.04	100	0	26.2 ± 5.3	21.1	1.00	100	0	13.9 ± 4.7	18.2	0.83	100	0	22.1 ± 3.4	22.2	0.00
Cry1A/Cry2A + Cry34/35Ab	100	0	12.3 ± 3.6	22.0	3.56	100	0	23.8 ± 6.3	30.5	1.69	100	0	3.1 ± 2.1	24.2	15.81[s]	100	0	12.8 ± 2.8	25.5	16.50[s]
Cry3B + Cry1F	100	0	33.9 ± 5.3	30.7	0.30	100	0	41.5 ± 5.3	40.3	0.04	100	0	33.9 ± 5.7	26.7	1.70	100	0	36.4 ± 3.1	33.6	0.69
Cry3B + Cry34/35Ab	100	0	29.2 ± 7.4	24.9	0.65	100	0	60.0 ± 4.5	58.2	0.09	100	0	36.9 ± 5.9	35.6	0.05	100	0	42.1 ± 4.0	38.5	1.04
Cry1F + Cry34/35Ab	100	0	30.8 ± 4.9	26.3	0.67	100	0	21.5 ± 4.8	24.9	0.39	100	0	24.6 ± 5.1	18.2	1.80	100	0	25.6 ± 2.8	23.4	0.55
Cry1A/Cry2A + Cry3B + Cry1F	100	0	13.9 ± 4.2	14.6	0.03	100	0	26.2 ± 5.3	20.4	1.33	100	0	12.3 ± 4.3	13.1	0.04	100	0	17.4 ± 2.8	16.5	0.12
Cry1A/Cry2A + Cry3B + Cry34/35Ab	100	0	6.2 ± 2.7	11.9	2.05	100	0	23.1 ± 6.3	29.5	1.29	100	0	1.5 ± 1.5	17.5	11.47[s]	100	0	10.3 ± 2.7	19.0	9.69[s]
Cry1A/Cry2A + Cry1F + Cry34/35Ab	100	0	7.7 ± 3.6	12.5	1.37	100	0	9.2 ± 4.3	12.7	0.71	100	0	1.5 ± 1.5	9.0	4.42[s]	100	0	6.2 ± 2.0	11.5	5.48[s]
Cry3B + Cry1F + Cry34/35Ab	100	0	21.5 ± 6.2	14.2	2.87	100	0	21.5 ± 4.8	24.2	0.25	100	0	23. 1± 5.0	13.1	5.68[s]	100	0	22.1 ± 3.0	17.4	2.94
Cry1A/Cry2A + Cry3B + Cry1F + Cry34/35Ab	100	0	4.6 ± 2.4	6.8	0.49	100	0	9.2 ± 4.3	12.3	0.57	100	0	1.5 ± 1.5	6.5	2.63	100	0	5.1 ± 1.8	8.6	2.99
Negative	---	100	9.2 ± 3.7	---	---	---	100	3.1 ± 3.1	---	---	---	100	4.6 ± 3.3	---	---	---	100	5.6 ± 1.9	---	---

[a] In each field trial, 5 individual kernels per ear with 10 ears (n = 50) were examined for ears of pure Bt maize plantings; 25 kernels per ear with 10 ears (n = 250) were tested for ears of pure non-Bt maize plantings; and for refuge ears in RIB, 5 individual kernels per ear with 13 ears (n =

65 for each trial and n = 195 for the pooled data) were assayed. Pure Bt: primary ears of pure Bt maize planting; pure non-Bt: primary ears of pure non-Bt maize planting; and RIB refuge: primary ears of the refuge plants in the RIB planting.

[b] O: observation frequency; and E: expected frequency based on the assumption of independent segregation. For example, expected frequency of Cry1A/Cry2A + Cry3B was calculated using the observed frequency of Cry1A/Cry2A timed by the observed frequency of Cry3B. Then χ^2 was determined using the equation: $\chi^2 = (n/100) [(O-E)^2/E + (E-O)^2/(100-E)]$.

[c] Pooled data across the three trials.

[s] Indicates significantly different from the assumption of independent segregation in χ^2-tests with df =1 at the $\alpha = 0.05$ level.

refuge ears did not significantly ($P > 0.05$) affect larval survival at the early insect stages (e.g., at 6-day after release of neonates).   For example, at 6-day of neonate release, larval survivorship for in-field observation was 62.3% on refuge ears and 61.2% on pure non-Bt ears (Table 6.2).   However, development of corn earworm on the RIB refuge ears was significantly ($P < 0.05$) delayed compared to that on the pure non-Bt ears.   Bt protein contamination delayed larval development by approximately one instar after 6 days of neonate release for in-field observation.   After 12 days as well as in subsequent observations, both larval survivorship and development were affected considerably ($P < 0.05$).   For example, at 18-day, survivorship on pure non-Bt corn ears was 43.9%, while on refuge ears it was only 16.2% (Table 6.2).   Similarly, compared to pure non-Bt corn ears, larval development after 12 days on refuge ears was significantly ($P < 0.05$) delayed by 1.5-instar for the in-field observations (Fig. 6.3B).

6.3.3 Lab Assay for Survival and Development of Corn Earworm

Similarly as observed in the in-field observation, the lab assay showed that the survivalship of corn earworm on the Bt corn ears was significantly ($P < 0.0001$) lower than that on the non-Bt ears from RIB and pure non-Bt plantings.   No corn earworm larvae survived to the pupal stage on the Bt corn ears (Table 6.3).   Lab assay also confirmed no significant effects ($P > 0.05$) of Bt protein contamination in refuge ears on larval survival at the early insect growth stages (Fig. 6.4A).   Larval survivorship for 6-day observation in the lab assay was 79.6% on refuge ears and 79.4% on pure non-Bt ears, and these values for 9-day were 57.2 and 61.1%, respectively (Fig. 6.4A and Table 6.3).   Nevertheless, the survivalship of corn earworm on the RIB refuge ears was significantly ($P < 0.05$) reduced

relative to that on pure non-Bt ears at 12-day as well as the subsequent observations, at the

pupal and adult stages (Fig. 6.4A).   Likewise, development of corn earworm on the RIB

refuge ears was significantly ($P < 0.05$) delayed by 1.0-2.0 instars compared to that on the

pure non-Bt ears (Fig. 6.4B and Table 6.4).   In the lab assay, 43.9 and 38.3% neonates on

pure non-Bt corn ears successfully developed to pupae and adults, respectively, while these

values on refuge ears were only 6.7 and 4.6%, which corresponded to a reduction of 84.7%

for pupation and 88.1% for adult emergence ($P < 0.05$) (Fig. 6.4A and Table 6.3).

Fig. 6.3. In-field observation on survivorship (A), and development (B) of *Helicoverpa zea* on ears of Genuity® SmartStax™ Bt and non-Bt maize plants in three planting patterns. Detailed data are reported in Table 6.2. Insect development was converted to development index: 1 = 1st instar, 2 = 2nd instar, …, 6 = 6th instar, 7 = pupal stage. Sample size for measuring survivorship was 240 larvae for RIB and 120 larvae for pure Bt and pure non-Bt. Sample size for determining larval development on pure non-Bt and RIB refuge was 55-150 larvae and on Bt plants was 1-13 larvae. Mean values within an observation time followed by a different letter were significantly different (Tukey's HSD test, α=0.05).
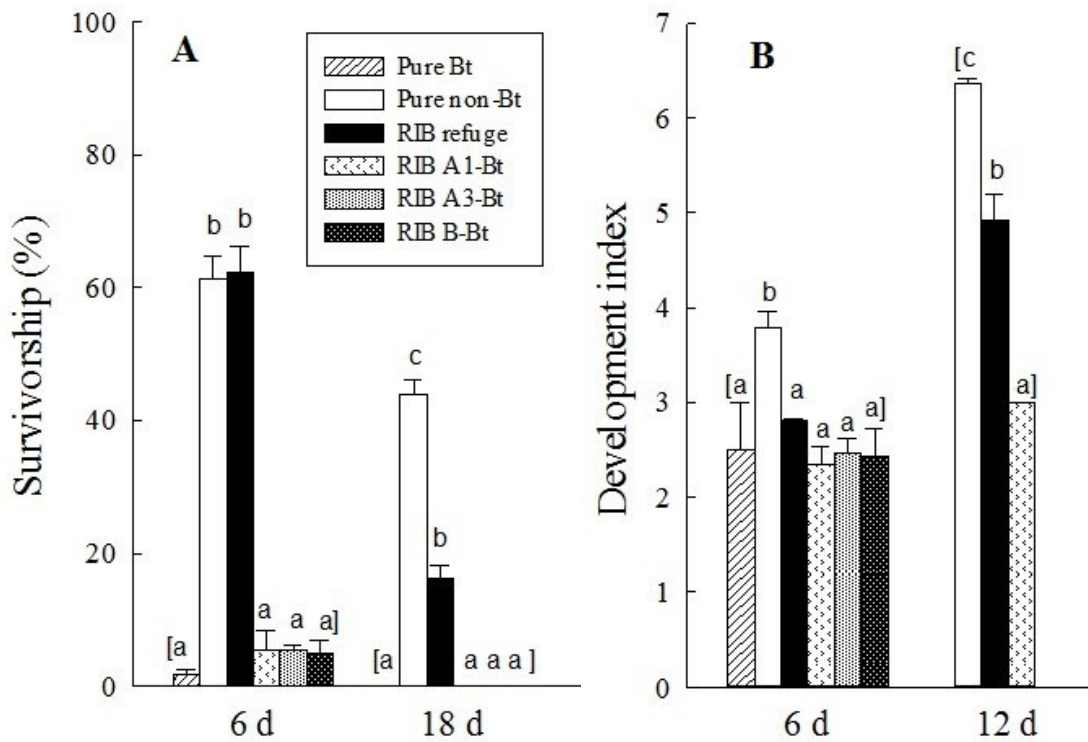
Table 6.2. In-field observation of survivorship and development (mean ± sem) of *Helicoverpa zea* on ears of Genuity$^®$ SmartStax^{TM} Bt and non-Bt maize plants in three planting patterns [a].

Ears		Survivorship (%)[b]					Development index[c]		
		6-d	9-d	12-d	15-d	18-d	6-d	9-d	12-d
Pure Bt		1.68 ± 0.82 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	2.50 ± 0.50 a	---	---
RIB	A1-Bt	5.42 ± 2.92 a	0.83 ± 0.42 a	0.42 ± 0.42 a	0.00 ± 0.00 a	0.00 ± 0.00 a	2.35 ± 0.18 a	3.50 ± 0.50 b	3.00 a
	A3-Bt	5.42 ± 0.83 a	0.83 ± 0.42 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	2.47 ± 0.15 a	2.50 ± 0.50 a	---
	B-Bt	5.00 ± 1.91 a	1.25 ± 1.25 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	2.44 ± 0.29 a	3.00 ab	---
	Refuge	62.27 ± 3.97 b	38.67 ± 1.88 b	24.77 ± 3.84 b	19.12 ± 1.77 b	16.16 ± 2.08 b	2.81 ± 0.01 a	3.90 ± 0.07 b	4.91 ± 0.29 b
Pure non-Bt		61.20 ± 3.61 b	53.77 ± 5.65 c	45.54 ± 6.56 c	43.87 ± 2.17 c	43.87 ± 2.17 c	3.78 ± 0.17 b	5.40 ± 0.15 c	6.36 ± 0.05 c
F-test	F-value	$F_{5, 10}$= 96.69	$F_{5, 10}$= 74.31	$F_{5, 10}$= 133.63	$F_{5, 10}$= 606.47	$F_{5, 10}$= 501.23	$F_{5, 9}$= 6.14	$F_{4, 4}$= 20.26	$F_{2, 2}$= 37.92
	P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.01	0.006	0.026

[a] Pure Bt: pure Bt maize planting; pure non-Bt: pure non-Bt maize planting; RIB refuge: the refuge plants in the RIB planting; A1-Bt: the Bt plants immediately adjacent and within the same row as the refuge plant in RIB planting; A3-Bt: the 3rd Bt plants on both sides of the refuge plant in the same row in RIB planting, and B-Bt: the closest Bt plants on both sides of the refuge plant in the two adjacent rows in RIB planting. Means were calculated based on three independent field trials (treated as a random factor). Sample size for each mean for measuring survivorship was 240 larvae for RIB and 120 larvae for pure Bt and pure non-Bt. Sample size for determining larval development on pure non-Bt and RIB refuge was 55-150 larvae and on Bt plants was 1-13 larvae. Means in a column followed by a different letter were significantly different (Tukey's HSD test, α = 0.05). Pure Bt: pure Bt maize planting; pure non-Bt: pure non-Bt maize planting; RIB refuge: the refuge plants in the RIB planting.

[b] Insect survivorship after 12 d was estimated based on the sum of the number of live larvae inside the ears and the holes bored by late instar larvae in the shoot bags.

[c] Insect development were converted to a development index: 1= 1st instar, 2= 2nd instar, …, 6= 6th instar, 7= pupal stage.

In addition, the limited survivors on refuge ears had significantly ($P < 0.05$) lower pupal mass (284.3 mg/pupa) and longer developmental time to become pupae (19.2 days) or adults (30.4 days) compared to the pupal mass (413.5 mg/pupa) and developmental times (13.9 days to pupa and 25.4 days to adult) on pure non-Bt corn ears (Fig. 6.4C and Table 6.4).

6.3.4 Field-Plus-Lab Assay for Survival and Development of Corn Earworm

Results of the field-plus-lab assay showed that at the time when ears were collected from field plants there were no significant ($P > 0.05$) differences in number of larvae per ear or larval development between pure non-Bt and RIB plantings (Fig. 6.5). However, over time, the number of live corn earworm on refuge ears decreased significantly ($P < 0.05$) and larval development on refuge ears was delayed significantly ($P < 0.05$) compared with the larvae on pure non-Bt corn ears (Fig. 6.5). For example, at 10-day after ears detached from plants, the number of live larvae on refuge ears was reduced by 54.2% and larval development delayed by 1.5-instar compared to pure non-Bt corn ears. Ultimately, the Bt protein contamination reduced pupation by 75.0%, pupal mass by 22.7%, and moth emergence rate by 80.5% (Fig. 6.5, Table 6.5). Results of the field-plus-lab assay validated that the RIB will be not effective in providing refuge populations for corn earworm.
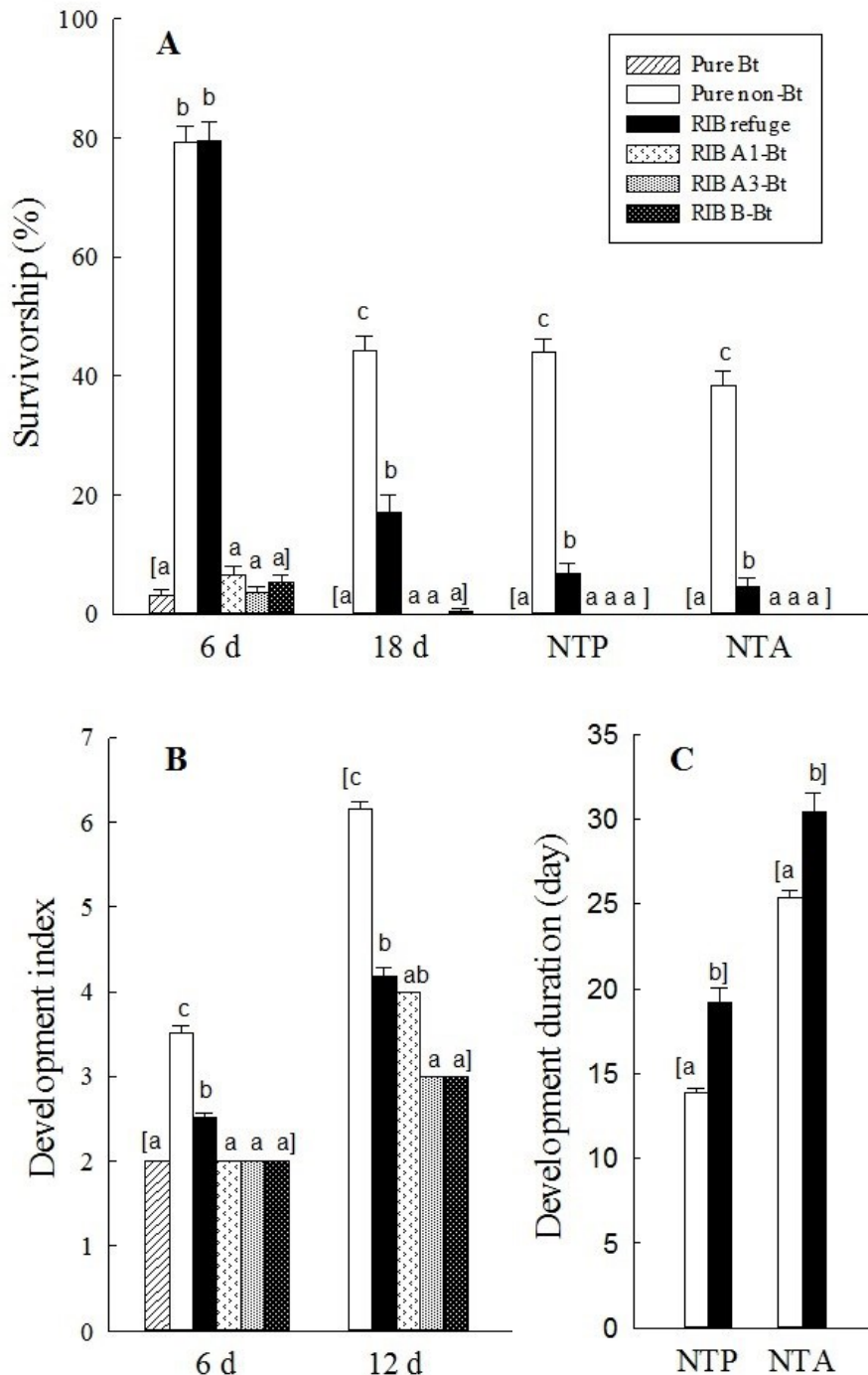
Fig. 6.4. Lab assay on survivorship (A), development (B), and development duration (C) of *Helicoverpa zea* on ears of Genuity® SmartStax™ Bt and non-Bt maize plants in three planting patterns. Detailed data are reported in (Table 6.3 and 6.4). Insect development was converted to development index: 1 = 1st instar, 2 = 2nd instar, …, 6 = 6th instar, 7 = pupal stage. NTP: neonate-to-pupa; NTA: neonate-to-adult. Means were calculated based on four independent assays (treated as a random factor). Sample size for each treatment mean was based on 300 larvae for measuring survivorship. Sample size for determining larval development was 122-239 larvae for pure non-Bt and RIB refuge, and 1-19 larvae for Bt plants. Mean values within an observation time followed by a different letter were significantly different (Tukey's HSD test, $\alpha = 0.05$).

Table 6.3. Lab assay on survivorship (mean ± sem) of *Helicoverpa zea* on ears of Genuity® SmartStax™ Bt and non-Bt maize plants in three planting patterns [a].

Assay[b]	Ears		Survivorship (%)						
			6-d	9-d	12-d	15-d	18-d	NTP[c]	NTA[d]
Lab assay-1	Pure Bt		6.67 ± 4.41 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
	RIB	A1-Bt	1.67 ± 1.67 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		A3-Bt	1.67 ± 1.67 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		B-Bt	3.33 ± 3.33 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		Refuge	73.67 ± 11.29 b	53.50 ± 8.54 b	41.70 ± 5.50 b	27.77 ± 2.77 b	22.93 ± 2.07 b	9.73 ± 5.02 b	4.17 ± 4.17 a
	Pure non-Bt		80.33 ± 12.25 b	64.83 ± 6.67 b	55.67 ± 3.20 c	44.40 ± 3.23 c	40.73 ± 4.91 c	40.73 ± 4.91 c	33.30 ± 6.41 b
	F-test	F-value	$F_{5,10}$= 19.22	$F_{5,10}$= 98.19	$F_{5,10}$= 227.65	$F_{5,10}$= 339.47	$F_{5,10}$= 194.05	$F_{5,10}$= 23.94	$F_{5,10}$= 18.73
		P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Lab assay-2	Pure Bt		1.25 ± 1.25 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
	RIB	A1-Bt	4.06 ± 1.39 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		A3-Bt	1.25 ± 1.25 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		B-Bt	5.94 ± 2.57 a	1.56 ± 1.56 a	1.56 ± 1.56 a	1.56 ± 1.56 a	1.56 ± 1.56 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		Refuge	80.63 ± 8.17 b	60.63 ± 5.43 b	47.50 ± 5.68 b	36.25 ± 3.35 b	25.63 ± 8.61 b	6.56 ± 4.72 b	6.56 ± 4.72 b
	Pure non-Bt		82.50 ± 3.06 b	66.88 ± 1.08 b	60.63 ± 4.80 c	48.44 ± 7.13 c	45.31 ± 5.60 c	45.31 ± 5.60 c	40.00 ± 6.63 c
	F-test	F-value	$F_{5,15}$= 58.85	$F_{5,15}$= 194.83	$F_{5,15}$= 110.41	$F_{5,15}$= 76.03	$F_{5,15}$= 32.19	$F_{5,15}$= 33.01	$F_{5,15}$= 25.95
		P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Lab assay-3	Pure Bt		2.50 ± 1.44 a	1.25 ± 1.25 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
	RIB	A1-Bt	7.50 ± 3.23 a	2.50 ± 1.44 a	1.25 ± 1.25 a	1.25 ± 1.25 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		A3-Bt	3.75 ± 1.25 a	1.25 ± 1.25 a	1.25 ± 1.25 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		B-Bt	7.50 ± 1.44 a	1.25 ± 1.25 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		Refuge	81.25 ± 3.75 b	57.50 ± 3.22 b	38.75 ± 3.75 b	25.00 ± 4.08 b	11.25 ± 3.15 b	5.00 ± 2.04 b	3.75 ± 1.25 b
	Pure non-Bt		81.25 ± 3.15 b	62.50 ± 1.44 b	51.25 ± 4.27 c	50.00 ± 4.56 c	50.00 ± 4.56 c	50.00± 4.56 c	42.50 ± 4.33 c

F-test	F-value	$F_{5, 15}= 79.01$	$F_{5, 15}= 71.00$	$F_{5, 15}= 83.58$	$F_{5, 15}= 91.75$	$F_{5, 15}= 167.77$	$F_{5, 15}= 104.32$	$F_{5, 15}= 107.17$
	P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Lab assay-4	Pure Bt		2.50 ± 1.44 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
	RIB	A1-Bt	11.25 ± 4.27 b	1.25 ± 1.25 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		A3-Bt	7.50 ± 1.44 ab	1.25 ± 1.25 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		B-Bt	3.75 ± 2.39 a	1.25 ± 1.25 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
		Refuge	81.25 ± 4.27 c	56.25 ± 7.18 b	35.00 ± 5.40 b	16.25 ± 4.27 b	10.00 ± 3.54 b	6.25 ± 2.39 b	3.75 ± 1.25 b
	Pure non-Bt		73.75 ± 4.27 c	51.25 ± 2.39 b	42.50 ± 2.50 c	40.00 ± 3.53 c	40.00 ± 3.53 c	38.75 ± 3.15 c	36.25 ± 3.15 c
F-test	F-value	$F_{5, 15}= 98.32$	$F_{5, 15}= 84.59$	$F_{5, 15}= 174.40$	$F_{5, 15}= 94.58$	$F_{5, 15}= 45.40$	$F_{5, 15}= 65.72$	$F_{5, 15}= 96.79$
	P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

[a] Treatment mean in Lab assay-1 was based on 60 larvae, while it was based on 80 larvae for the rest three assays. Means in a column within a lab assay followed by a different letter were significantly different (Tukey's HSD test, α=0.05). Pure Bt: pure Bt maize planting; pure non-Bt: pure non-Bt maize planting; RIB refuge: the refuge plants in the RIB planting; A1-Bt: the Bt plants immediately adjacent and within the same row as the refuge plant in RIB planting; A3-Bt: the 3rd Bt plants on both sides of the refuge plant in the same row in RIB planting, and B-Bt: the closest Bt plants on both sides of the refuge plant in the two adjacent rows in RIB planting.

[b] Lab assay-1 contained three replications with 8-10 ears per replication, while the rest three assays consisted of four replications with 8-10 ears per replication.

[c] NTP: neonate to pupa.

[d] NTA: neonate to adult.

Table 6.4. Lab assay on development index, pupal weight, and development time (mean ± sem) of *Helicoverpa zea* on ears of Genuity[®] SmartStax[TM] Bt and non-Bt maize plants in three planting patterns [a].

Trial	Ears		Development index[b]				Pupal mass and development duration			
			6-d	9-d	12-d	15-d	No. pupa	Pupal mass (mg/pupa)	NTP[c]	NTA[d]
Lab assay-1	Pure Bt		2.00 a	---	---	---	---	---	---	---
	RIB	A1-Bt	2.00 a	---	---	---	---	---	---	---
		A3-Bt	2.00 a	---	---	---	---	---	---	---
		B-Bt	2.00 a	---	---	---	---	---	---	---
		Refuge	2.68 ± 0.11 b	3.32 ± 0.09 a	4.04 ± 0.20 a	4.67 ± 0.22 a	4	264.2 ± 29.2 a	20.75 ± 1.60 b	32.50 ± 3.50 b
	Pure non-Bt		3.17 ± 0.07 c	5.02 ± 0.21 b	6.00 ± 0.10 b	6.66 ± 0.13 b	22	409.7 ± 7.4 b	14.55 ± 0.38 a	26.94 ± 0.37 a
	F-test	F-value	$F_{5,3}$= 30.88	$F_{1,2}$= 379.76	$F_{1,2}$= 62.25	$F_{1,2}$= 49.79		$F_{1,22}$= 55.19	$F_{1,22}$= 26.32	$F_{1,16}$= 13.08
		P-value	0.009	0.003	0.016	0.020		< 0.0001	<0.0001	0.002
Lab assay-2	Pure Bt		2.00 a	---	---	---	---	---	---	---
	RIB	A1-Bt	2.00 ± 0.00 a	---	---	---	---	---	---	---
		A3-Bt	2.00 a	---	---	---	---	---	---	---
		B-Bt	2.00 ± 0.00 a	2.00 a	3.00 a	---	---	---	---	---
		Refuge	2.39 ± 0.13 a	3.19 ± 0.15 b	3.79 ± 0.17 a	4.22 ± 0.19 a	5	283.3 ± 30.6 a	21.00 ± 1.30 b	32.40 ± 1.44 b
	Pure non-Bt		3.41 ± 0.10 b	5.04 ± 0.08 c	5.89 ± 0.06 b	6.51 ± 0.15 b	32	437.3 ± 11.3 b	14.88 ± 0.33 a	26.76 ± 0.34 a
	F-test	F-value	$F_{5,6}$= 51.98	$F_{2,3}$= 101.13	$F_{2,3}$= 72.84	$F_{1,3}$= 76.18		$F_{1,32}$= 23.93	$F_{1,32}$= 32.96	$F_{1,29}$= 28.31
		P-value	< 0.0001	0.002	0.003	0.003		< 0.0001	< 0.0001	0.002
Lab assay-3	Pure Bt		2.00 ± 0.00 a	2.00 a	---	---	---	---	---	---
	RIB	A1-Bt	2.00 ± 0.00 a	2.50 ± 0.50 a	4.00 b	---	---	---	---	---
		A3-Bt	2.00 ± 0.00 a	2.00 a	3.00 a	---	---	---	---	---
		B-Bt	2.00 ± 0.00 a	3.00 ab	---	---	---	---	---	---

		Refuge	2.60 ± 0.03 b	3.35 ± 0.06 b	4.35 ± 0.12 c	5.14 ± 0.15 a	4	309.0 ± 33.3 a	17.75 ± 1.31 b	29.00 ± 2.08 b
	Pure non-Bt		3.64 ± 0.20 c	5.43 ± 0.22 c	6.49 ± 0.18 d	6.95 ± 0.03 b	40	412.4 ± 6.8 b	12.85 ± 0.21 a	23.62 ± 0.28 a
	F-test	F-value	$F_{5,10}=361.40$	$F_{5,4}=26.15$	$F_{3,3}=1041.87$	$F_{1,3}=115.09$		$F_{1,39}=25.94$	$F_{1,39}=37.47$	$F_{1,32}=23.27$
		P-value	<0.0001	0.004	<0.0001	0.002		<0.0001	<0.0001	<0.0001
Lab assay-4	Pure Bt		2.00 ± 0.00 a	---	---	---	---	---	---	---
	RIB	A1-Bt	2.00 ± 0.00 a	3.00 b	---	---	---	---	---	---
		A3-Bt	2.00 ± 0.00 a	3.00 b	---	---	---	---	---	---
		B-Bt	2.00 ± 0.00 a	2.00 a	---	---	---	---	---	---
		Refuge	2.48 ± 0.04 b	3.53 ± 0.05 c	4.53 ± 0.18 a	5.33 ± 0.14 a	5	277.5 ± 16.2 a	17.40 ± 0.60 b	29.00 ± 1.53 b
	Pure non-Bt		3.74 ± 0.07 c	5.71 ± 0.04 d	6.24 ± 0.08 b	6.94 ± 0.03 b	31	393.4 ± 7.2 b	13.26 ± 0.25 a	24.62 ± 0.30 a
	F-test	F-value	$F_{5,10}=361.40$	$F_{4,3}=522.12$	$F_{1,3}=58.41$	$F_{1,3}=149.21$		$F_{1,31}=45.16$	$F_{1,31}=30.08$	$F_{1,27}=15.60$
		P-value	<0.0001	0.0001	0.005	0.001		<0.0001	<0.0001	0.0005

[a] Pure Bt: pure Bt maize planting; Pure non-Bt: pure non-Bt maize planting; RIB refuge: the refuge plants in the RIB planting; A1-Bt: the Bt plants immediately adjacent and within the same row as the refuge plant in RIB planting; A3-Bt: the 3rd Bt plants on both sides of the refuge plant in the same row in RIB planting, and B-Bt: the closest Bt plants on both sides of the refuge plant in the two adjacent rows in RIB planting. Sample size for each mean for measuring development index was 16-66 larvae for pure non-Bt and RIB refuge and 1-9 larvae for Bt plants. Means in a column within a lab assay followed by a different letter were significantly different (Tukey's HSD test, $\alpha=0.05$).

[b] Insect development were converted to development index: 1= 1st instar, 2= 2nd instar, …, 6= 6th instar, 7= pupal stage.

[c] NTP: neonate-to-pupa development time (d).

[d] NTA: neonate-to-adult development time (d).

Table 6.5. Field-plus-lab assay on pupal mass (mean ± sem) of *Helicoverpa zea* on ears of RIB refuge and pure non-Bt plants [a].

Ears	Total No. pupa	Pupal weight (mg)
RIB refuge	41	334.7 ± 13.6 a
Pure non-Bt	227	433.2 ± 5.1 b
F-test F-value	---	$F_{1, 2}$= 52.14
P-value	---	0.02

[a] Means in a column followed by a different letter were significantly different (Tukey's HSD test, α=0.05). RIB refuge: the refuge plants in the RIB planting, and pure non-Bt: pure non-Bt maize planting.
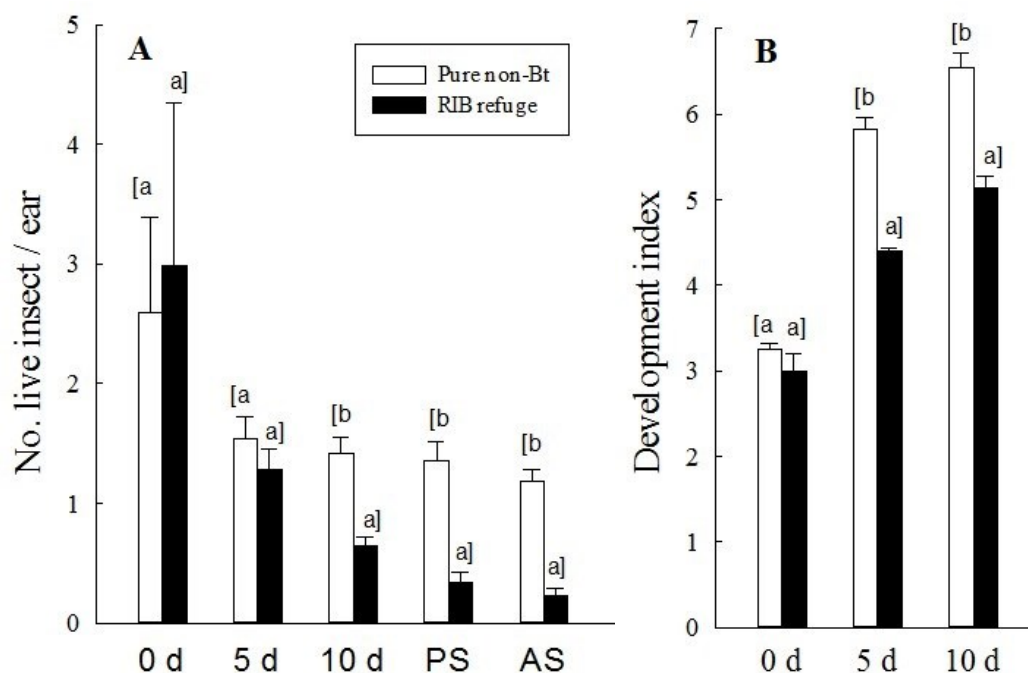
Fig. 6.5. Field-plus-lab assay on occurrence (A), and development (B) of *Helicoverpa zea* on ears of RIB refuge and pure non-Bt plants. Pure non-Bt: pure non-Bt maize planting; and RIB refuge: the refuge plants in the RIB planting. Insect development was converted to development index: 1 = 1st instar, 2 = 2nd instar, …, 6 = 6th instar, 7 = pupal stage. PS: pupal stage; AS: adult stage. 0 d = the day that ears were sampled from fields. Means were calculated based on three independent assays (treated as a random factor). Sample size for each treatment mean was 160 ears for larval occurrence. Sample size for determining larval development was 104-479 larvae. Mean values within an observation time followed by a different letter were significantly different (Tukey's HSD test, α = 0.05).

6.4 Discussion

Multiple field trials and laboratory assays were conducted to assess the impacts of pollen contamination on survival and development of corn earworm in seed mixture plantings. The overall results were consistent across three study methods including in-field observation, lab assay and field-plus-lab assay (Figs. 6.3-6.5), as well as across all trials within each study method (Tables 6.2-6.5). The data clearly showed that the high levels of Bt protein contamination in refuge ears in RIB significantly affected larval survival, growth, and development of corn earworm, suggesting the currently used RIB approach in the U.S. is not effective for providing refuge populations of corn earworm for resistance management.

In this study, qualitative ELISA tests were used to test the protein expression in kernels of different corn ears. However, the concentration of each Bt protein in individual kernels was not measured because of cost. Studies have shown that production of Bt proteins is typically dominant in Bt plants and thus concentration in the refuge kernels is expected to have been high (Chilcutt and Tabashnik, 2004). Furthermore, the strong bands exhibited in the ELISA strips (Fig. 6.1) also indicated that the Bt protein expression levels were not low.

The results of the current study showed that the four Bt protein groups segregated independently in the Genuity$^®$ SmartStax^{TM} trait. In addition, it has been commonly assumed that, for a particular gene in pure Bt corn planting of a corn hybrid (F_1), 25% F_2 kernels should be homozygous and 50% should be hemizygous for the Bt allele, while the remaining 25% should not express the Bt protein (Chilcutt et al., 2007; Chilcutt and Tabashnik, 2004; Burkness et al., 2010; 2011). Contrary to this common assumption, our results showed 100% F_2 corn kernels of a pure-planted Bt corn hybrid containing Genuity$^®$

SmartStax^{TM} trait expressed all the Bt proteins, suggesting that alleles of all Bt genes in Genuity® SmartStax^{TM} are likely homozygous in the two parents of the F$_1$ corn hybrids or a more complicate gene structure and inheritance can be associated with the Bt corn trait (Randolph, 1936; Kowles and Phillips, 1985; Schweizer et al., 1995; Trifa and Zhang, 2004). Corn kernels are mainly made of three components: tegument, embryo and endosperm with endosperm accounting for 80-90% of the total weight (Trifa and Zhang, 2004).   The embryos are diploid, emerging from the fusion of one haploid maternal nucleus and one haploid male nucleus (Randolph, 1936; Trifa and Zhang, 2004).   The teguments are diploid and composed wholly of maternal origin.   However, the endosperms are triploid, resulting from the fusion of two maternal polar nuclei with one sperm nucleus (Schweizer et al., 1995; Kowles and Phillips, 1985).   Therefore, the gene inheritance in the corn kernels might be not as simple as one copy from paternal nucleus and one copy from maternal nucleus.   A transgenic corn kernel might have different transgene copies depending on the parent and the pollination event.   Similarly in another independent study, we also found that 100% F$_2$ corn kernels of a pure-planted Bt corn hybrid containing Agrisure® Viptera^{TM} 3111 trait expressed both the Vip3A and Cry1Ab proteins.   The results of these studies suggest that the 'homozygous' or 'complicated genetic' property of Bt genes may commonly exist in different Bt corn products.

Previous reports indicated that Bt proteins could decrease with time in excised leaf tissue of cotton and corn, but the biological activity was maintained for at least several days (Kranthi, 2006; Huang et al., 2007; Poongothai et al., 2013).   We expected that the biological activity in detached corn ears should be maintained much longer than in detached

leaf tissue because corn ears can be preserved considerably longer than leaf tissue. The similar results observed across the in-field observations, lab assays, and field-plus-lab assays suggest that the protocols used in the current study were appropriate. Nevertheless, if Bt degradation after ears are detached is significant, the effect of cross-pollination of intact plants on insect populations could be greater than that observed in this study. In addition, reproduction of many lepidopteran species is proportional to the nutrient reserves acquired during larval stages and is correlated with pupal weight (Leahy and Andow, 1994). Thus, the reduced pupal weight plus delayed development of corn earworm feeding on refuge ears suggest that cross-pollination could have additional effects on the adult reproduction.

Corn earworm is considered to be distributed throughout the U.S. except for Alaska (Capinera, 2000). In the south, corn earworm are known to overwinter in the pupal stage, but it usually cannot overwinter in most areas of the U.S. Corn Belt especially north of 40-degrees latitude due to lethal periods of freezing temperatures during the winter season (Hardwick, 1965; Lindgren et al., 1994; Sandstorm et al., 2007; Morey et al., 2012). Therefore, corn earworm is commonly believed to have a one-way migration to northward corn-growing regions from southern overwintering sites every year (US-EPA, 2004). Thus, the considerable effect of Bt protein contamination caused by cross-pollination on refuge corn earworm is not necessary to imply any deficiency of the RIB strategy for managing this pest in the U.S. Corn Belt.

Some indirect evidences also indicated that corn earworm might undergo a reverse migration from some northern corn-growing regions to the southern regions (Gould et al., 2002; Westbrook, 2008), but details of this phenomenon are still unknown. In theory, if

reverse migration of corn earworm is present, the selection pressure for resistance

development would be accelerated because migration populations would contribute some

resistance genes to the local populations.

In conclusion, results of the comprehensive field and laboratory studies suggest that RIB

for Bt corn at the levels at which it is currently implemented is unlikely to provide adequate

refuge populations for ear-feeding targets such as corn earworm.   Effective refuge strategies

must be built upon appropriate analyses of all key pests and require different approaches in

different regions.

6.5 References

Abendroth, L.J., Elmore, R.W., Boyer, M.J., Marlay, S.K., 2011. Corn growth and development. Ames, IA: PMR 1009, Iowa State University Extension.

Alyokhin, A., 2011. Scant evidence supports EPA's pyramided Bt corn refuge size of 5%. Nat. Biotech. 29, 577-578.

Bannert, M., 2006. Simulation of transgenic pollen dispersal by use of different grain colour maize. Ph.D. Dissertation, Swiss Federal Institute of Technology Zurich. Available at: http://www.agrisite.de/doc/ge_img/pollen-swiss.pdf.

Burkness, E.C., Dively, G., Patton, T., Morey, A.C., Hutchison, W.D., 2010. Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae) under field conditions. GM crops 1, 1-7.

Burkness, E.C., Hutchison, W.D., 2012. Bt pollen dispersal and Bt kernel mosaics: integrity of non-Bt refugia for lepidopteran resistance management in maize. J. Econ. Entomol. 105, 1477-1870.

Burkness, E.C., O'Rourke, P.K., Hutchison, W.D., 2011. Cross-pollination of nontransgenic corn ears with transgenic Bt corn: efficacy against lepidopteran pests and implications for resistance management. J. Econ. Entomol. 104, 1476-1479.

Capinera, J.L., 2000. Corn earworm, *Helicoverpa* (=*Heliothis*) *zea* (Boddie) (Lepidoptera: Noctuidae). Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. EENY-145 (IN302).

Carroll, M.W., Head, G., Caprio, M., 2012.When and where a seed mix refuge makes sense for managing insect resistance to Bt plants. Crop Protect. 38, 74-79.

Carroll, M.W., Head, G., Caprio, M., Stork, L., 2013. Theoretical and empirical assessment of a seed mix refuge in corn for southwestern corn borer. Crop Protect. 49, 58-65.

Chilcutt, C.F., Odvody, G.N., Correa, J.C., Remmers, J., 2007. Effects of *Bacillus thuringiensis* transgenic corn on corn earworm and fall armyworm (Lepidoptera: Noctuidae) densities. J. Econ. Entomol. 100, 327-334.

Chilcutt, C.F., Tabashnik, B.E., 2004. Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maize. Proc. Natl. Acad. Sci. U.S.A. 101, 7526-7529.

Davis, P.M., Onstad, D.W., 2000. Seed mixtures as a resistance management strategy for European corn borers (Lepidoptera: Crambidae) infesting transgenic corn expressing Cry1Ab protein. J. Econ. Entomol. 93, 937-948.

Dhurua, S., Gujar, G.T., 2011. Field-evolved resistance to Bt protein Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) from India. Pest Manag. Sci. 67, 898-903.

Difonzo, C., Collen, E., 2012. Handy Bt trait table. Available at: http://www3.ag.purdue.edu/agry/PCPP/Documents/Entry%20forms/Handy_Bt_Trait_Table.pdf.

Edgerton, M.D, Fridgen, J., Anderson-Jr, J.R., Ahlgrim, J., Criswell, M., Dhungana, P., Gocken, T., Li, Z., Mariappan, S., Pilcher, C.D., Rosielle, A., Stark, S.B., 2012. Transgenic insect resistance traits increase corn yield and yield stability. Nat. Biotechnol. 30, 493-496.

Gassmann, A.J., Petzold-Maxwell, J.L., Keweshan, R.S., Dunbar, M.W., 2011. Field-evolved resistance to Bt maize by western corn rootworm. PloS ONE 6, e22629.

Gould, F., 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Ann. Rev. Entomol. 43, 701-726.

Gould, F., Blair, N., Reid, M., Rennie, T.L., Micinski, S., 2002. *Bacillus thuringiensis*-toxin resistance management: stable isotope assessment of alternate host use by *Helicoverpa zea*. Proc. Natl. Acad. Sci. U.S.A. 99, 16581-16586.

Hardwick, D.F., 1965.The corn earworm complex. Mem. Entomol. Soc. Can. 40, 1-246.

Huang, F., Andow, D.A., Buschman, L.L., 2011. Success of the high dose/refuge resistance management strategy after 15 years of Bt crop use in North America. Entom. Exp. App. 140, 1-16.

Huang, F.N., Leonard, B.R., Andow, D.A., 2007. F_2 screen for resistance to a *Bacillus thuringiensis*-maize hybrid in the sugarcane borer (Lepidoptera: Crambidae). Bull. Entomol. Res. 97, 1-8.

Hutchison, W.D., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S.J., Abrahamson, M., Hamilton, K.L., Steffey, K.L., Gray, M.E., et al., 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt mazie growers. Science, 222-225.

James, C., 2013. Global status of commercialized biotech/GM crops: 2013. *ISAAA Brief* No. 46. ISAAA: Ithaca, NY, USA.

Kang, J., Onstad, D.W., Hellmich, R.L., Moser, S.E., Hutchison, W.D., Prasifka, J.R., 2012. Modeling the impact of cross-pollination and low toxin expression in corn kernels on adaptation of European corn borer (Lepidoptera: Crambidae) to transgenic insecticidal corn. Environ. Entomol. 41, 200-211.

Kowles, R.V., Phillips, R.L., 1985. DNA amplification patterns in maize endosperm nuclei during kernel development. Proc. Natl. Acad. Sci. U.S.A. 82, 7010-7014.

Kranthi, K.R., 2006. On the variability of Cry1Ac expression in commercialized *Bt* cotton varieties in India. Curr. Sci. 90, 1170-1172.

Leahy, T.C., Andow, D.A., 1994. Egg weight, fecundity, and longevity are increased by adult feeding in *Ostrinia nubilalis* (Lepidoptera: Pyralidae). Ann. Entomol. Soc. Am. 87, 342-349.

Lindgren, P.D., Westbrook, J.K., Bryant, V.M., Raulston, J.R., Esquivel, J.F., Jones, G.D., 1994. Origin of corn earworm (Lepidoptera: Noctuidae) migrants as determined by citrus pollen markers and synoptic weather systems. Environ. Entomol. 23, 562-570.

Mallet, J., Porter, P., 1992. Preventing insect adaptation to insect-resistant crops are seed mixtures or refugia the best strategy? Proc. R. Soc. Lond. B. Biol. Sci. 250,165-169.

Matten, S.R., Frederick, R.J., Reynolds, A.H., 2012. United States Environmental Protection Agency insect resistance management programs for plant-incorporated protectants and use of simulation modeling. pp 175-267. In: C.A. Wozniak, A. McHughen (eds), "Regulation of agricultural biotechnology: The United States and Canada". Springer.

Monsanto, 2012. IRM grower guide: insect resistance management for U.S. corn and cotton-growing areas. Available at: http://www.monsanto.com/products/Pages/insect-resistance-management.aspx.

Morey, A.C., Hutchison, W.D., Venette, R.C., Burkness, E.C., 2012. Cold hardiness of corn earworm (Lepidoptera: Noctuidae) pupae. Environ. Entomol. 41, 172-179.

NASS (National Agricultural Statistics Service), 2013. Acreage. USDA, Washington DC. USA. Available at: http://usda01.library.cornell.edu/usda/current/Acre/Acre-06-28-2013.pdf.

Onstad, D.W., Mitchell, P.D., Hurley, T.M., Lundgren, J.G., Porter, R.P., Krupke, C.H., Spencer, J,L., DiFonzo, C.D., Baute, T.S., Hellmich, R.L., Buschman, L.L., Hutchison, W.D., Tooker, J.F., 2011. Seeds of change: corn seed mixtures for resistance management and IPM. J. Econ. Entomol. 104, 343-352.

Ostlie, K.R., Hutchison, W.D. & Hellmich, R.L. (1997). Bt corn & European corn borer: long term success through resistance management, North Central Regional Extension Publication NCR 602.

Poongothai, S., Ilavarasan, R., Karrunakaran, C.M., 2013. Cry 1Ac levels and biochemical variations in Bt cotton as influenced by tissue maturity and senescence. Int. J. Biol. Res. 1, 49-55.

Randolph, L.F., 1936. Developmental morphology of the caryopsis in maize. J. Agric. Res. 53, 881-916.

Roush, R.T., 1998. Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? *Phil Trans R Soc Lond B* 353, 1777-1786.

Sandstrom, M.A., Changnon, D., Flood, B.R., 2007. Improving our understanding of *Helicoverpa zea* migration in the Midwest: assessment of source populations. *Plant Health Prog*. Available at: http://www.plantmanagementnetwork.org/pub/php/symposium/hzea/migrate/

SAS Institute, 2010. SAS/ STAT User's Third Edition, SAS Institute Inc, Cary, NC, USA.

Schweizer, L., Yerk-Davis, G.L., Phillips, R.L., Srienc, F., Jones, R.J., 1995. Dynamics of maize endosperm development and DNA endoreduplication. Proc. Natl. Acad. Sci. U.S.A. 92, 7070-7074.

Siebert, M.W., Nolting, S.P., Hendrix, W., Dhavala, S., Craig, C., Leonard, B.R., Stewart, S.D., All, J., Musser, Buntin, F.R., Samuel, L., 2012. Evaluation of corn hybrids expressing Cry1F, Cry1A.105, Cry2Ab2, Cry34Ab1/Cry35Ab1, and Cry3Bb1 against southern United States insect pests. J. Econ. Entomol. 105, 1825-1834.

Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W., Huckaba, R.M., 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 103, 1031-1038.

Tabashnik, B.E., Brévault, T., Carrière, Y., 2013. Insect resistance to Bt crops: lessons from the first billion acres. Nat. Biotechnol. 31, 510-521.

Trifa, Y., Zhang, D., 2004. DNA content in embryo and endosperm of maize kernel (*Zea mays* L.): impact on GMO quantification. J. Agric. Food Chem. 52, 1044-1048.

US-EPA (U.S. Environmental Protection Agency), 2001. Biopesticide registration action document: *Bacillus thuringiensis* plant-incorporated protectants. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm.

US-EPA (U.S. Environmental Protection Agency), 2004. EPA review of corn earworm/cotton bollworm (*Helicoverpa zea*) north-south migration and its impact on resistance management for Bt crops. Available at: http://www.epa.gov/scipoly/sap/meetings/2004/june/cbwreversemigration.pdf

US-EPA (U.S. Environmental Protection Agency), 2010a. Terms and conditions for Bt corn registrations 30 Sept 2010. Office of Pesticide Programs, Washington, DC. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/bt-corn-terms-conditions.pdf.

US-EPA (U.S. Environmental Protection Agency), 2010b. Biopesticides registration action document: *Bacillus thuringiensis* Cry1A.105 and Cry2Ab2 insecticidal proteins and the genetic material necessary for their production in corn. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/mon-89034-brad.pdf.

Van-Rensburg, J.B.J., 2007. First report of field resistance by stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. S. Afr. J. Plant Soil. 24, 147-151.

Westbrook, J.K., 2008. Noctuid migration in Texas within the nocturnal aeroecological boundary layer. Integr. Comp. Biol. 48, 99-106.

Yang, F., Kerns, D.L., Head, G.P., Leonard, B.R., Niu, Y., Huang, F., 2014. Occurrence, distribution, and ear damage of *Helicoverpa zea* (Lepidoptera: Noctuidae) in mixed plantings of non-Bt and Bt corn containing Genuity® SmartStax™ traits. Crop Protect. 55, 127-132.

Zar, J.H., 1984. Biostatistical analysis, 2nd ed. Prentice-Hall, Englewood Cliffs, NJ.

Zhao, J.Z., Cao, J., Li, Y., Collins, H.L., Roush, R.T., Earle, E.D., Shelton, A.M., 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat. Biotechnol. 21, 1493-1497.

CHAPTER 7. SUMMARY AND CONCLUSIONS

Transgenic crops (corn and cotton) expressing *Bacillus thuringiensis* (Bt) proteins for pest control have been planted on >178 million acres in the world in 2013. However, the intensive use of Bt crops places a high selection pressure on the target pest populations that could accelerate evolution of resistance. To delay resistance development, a 'high dose/refuge' strategy has been adopted for planting Bt corn in the U.S. and several other countries. Before 2010, the refuge was required to be arranged in a structured form that was implemented as blocks or strips of non-Bt crops to maintain susceptible insect populations. Since 2010, a seed mixture refuge strategy of 95: 5% (Bt: non-Bt corn seeds), also called RIB (refuge-in-the-bag) has been adopted for planting pyramided Bt corn in the U.S. Corn Belt. Pyramided Bt corn contains two or more Bt genes targeting a same pest species. The corn earworm, *Helicoverpa zea* (Boddie), and fall armyworm, *Spodoptera frugiperda* (J.E. Smith), are two major target pests of pyramided Bt corn in the U.S. Largely due to the lack of support data, the RIB strategy has not been approved in the south region of the U.S., where Bt cotton is also planted. Three of the major concerns for use of a seed mixture refuge strategy in the southern region are 1) the new pyramided Bt corn hybrids may not produce a high dose for the more Bt-tolerant pest species in the south (e.g. corn earworm, fall armyworm); 2) larval movement of target pest populations in RIB plantings may create a faviorable condition for resstance development; 3) the cross-pollination property of corn hybrids that can cause Bt protein contamination to refuge corn kernels in RIB plantings and the Bt protein contamination in RIB plantings could negatively affect (e.g. survival, growth, and development) the refuge insects, especially for ear feeders (e.g. corn earworm).

Currently, little information is available to address these concerns.   In this study, multiple field and laboratory tests were designed to: 1) determine the susceptibility to pyramided Bt corn in field populations of fall armyworm; 2) investigate occurrence and plant injury of corn earworm in mixed plantings of Bt and non-Bt corn in open fields; and 3) evaluate the intensity of Bt protein contamination in RIB and its associated effects on refuge populations of the ear feeder, corn earworm.

During 2011, a total of 150 F_2 two-parent families were established using single-pairing of feral fall armyworm collected from three locations in Louisiana and Florida. Susceptibility of these F_2 two-parent families to three commonly used pyramided Bt corn traits, Genuity[®] VT Double Pro[TM], Genuity[®] SmartStax[TM] and Agrisure[®] Viptera[TM] 3111 was examined in leaf tissue bioassay in the laboratory and whole plant tests in the greenhouse. The leaf tissue bioassay showed that none of these family lines survived for 7 days on Agrisure[®] Viptera[TM] 3111, while nine out of 149 families showed a less susceptibility to the leaf tissue of Genuity[®] VT Double Pro[TM] or Genuity[®] SmartStax[TM] plants.   Larvae of these nine families exhibited significant survivorship and growth on leaf tissue of the Bt corn plants. However, progeny of the survivors in the leaf tissue bioassays could not survive on whole plants of their corresponding Bt corn products in the greenhouse, suggesting these families were not resistant to the pyramided Bt corn traits.   These results suggest that the pyramided Bt corn products containing Genuity[®] VT Double Pro[TM], Genuity[®] SmartStax[TM], and Agrisure[®] Viptera[TM] 3111corn traits are effective against fall armyworm.

Multiple field trials were conducted during 2011-2012 to evaluate the occurrence, distribution, and ear damage of corn earworm in three planting patterns of non-Bt and Bt corn

plants containing Genuity® SmartStax™ and Agrisure® Viptera™ 3111 traits.   The three planting patterns were 1) pure stands of 27 Bt plants; 2) pure stands of 27 non-Bt plants; and 3) a mixed planting of one non-Bt plant in the center surrounded by 26 Bt plants.   In the field, egg populations of corn earworm were distributed randomly or uniformly, and the number of eggs laid was similar between Bt and non-Bt corn ears regardless of the planting patterns and corn products.   The results suggest that females of corn earworm have no egg-laying preference between Bt and non-Bt plants.   Pyramided Bt corn hybrids containing Genuity® SmartStax™ or Agrisure® Viptera™ 3111 traits were highly effective for corn earworm control with virtually no larvae or ear damage on the ears in pure stands of Bt corn and mixed plantings.   Larval occurrence (3rd - 5th instars) and ear damage on the refuge ears in mixed plantings were similar to or greater than that found on ears of pure stands of non-Bt plants.   However, larval development on refuge ears in mixed planting was significantly delayed relative to that on ears of pure non-Bt corn plantings.

During 2012-2013, multiple field trials and laboratory assays were conducted using the pyramided Bt corn containing the Genuity® SmartStax™ trait to assess the impacts of pollen contamination on survival and development of corn earworm in a RIB of 95% Bt and 5% non-Bt corn.   The results demonstrated that the currently adopted 95:5% RIB approach is inappropriate for providing refuge populations of corn earworm.   Cross-pollination in RIB caused majority (> 90%) of the refuge kernels to express ≥ one Bt protein.   The intensive Bt protein contamination in the refuge ears reduced neonate-to-adult survivorship to only 4.6%, a reduction of 88.1% relative to the larvae feeding on ears of pure non-Bt corn plantings.   In addition, the limited survivors on refuge ears had a lighter pupal mass and took longer

141

developmental time to become pupae and adults.   In conclusion, results of the

comprehensive field and laboratory studies suggest that the 95: 5% RIB cannot provide

adequate refuge populations for ear-feeding targets such as corn earworm.   Data generated

from this study should provide useful information for developing appropriate resistance

management strategies for the sustainable use of the Bt corn technology as a pest

management tool.

APPENDIX: LETTERS OF PERMISSION

A.1. Letter of Permission for Chapter 2

Permission Request

Waldemar Klassen <editor.flaentsoc@gmail.com>
Wed 10/1/2014 10:53 AM

Mark as unread

Dear Mr. Yang:

Yes, you are at liberty to reprint the article below in your thesis.

The Florida Entomological Society does not copyright articles published in the Florida Entomologist.

All published articles remain the intellectual property of the author(s).

Best wishes for a successful thesis defense.

Sincerely,

Waldemar Klassen, Editor

"SUSCEPTIBILITY OF LOUISIANA AND FLORIDA POPULATIONS OF *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTUIDAE) TO PYRAMIDED BT CORN CONTAINING GENUITY® VT DOUBLE PRO™ AND SMARTSTAX™
TRAITS" has been published in the Florida Entomologist (2013 vol. 96(3): 714-723).

← REPLY   ← REPLY ALL   → FORWARD   ⋯

Yang, Fei
Mon 9/29/2014 4:38 PM
已发送邮件

Mark as unread

To: ☐Waldemar Klassen <editor.flaentsoc@gmail.com>;

Dear Dr. Waldemar Klassen,

The following article "SUSCEPTIBILITY OF LOUISIANA AND FLORIDA POPULATIONS OF *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTUIDAE) TO PYRAMIDED BT CORN CONTAINING GENUITY® VT DOUBLE PRO™ AND SMARTSTAX™
TRAITS" has been published in the Florida Entomologist (2013 vol. 96(3): 714-723). The data from this manuscript will also be published as a chapter of my dissertation from Louisiana State University. This dissertation will be submitted to the graduate school this coming November. Could I request a letter or email stating that Florida Entomologist gives me permission to reprint the data from this manuscript for my dissertation? Thanks a lot.

Fei Yang

A.2. Letter of Permission for Chapter 3

ELSEVIER LICENSE TERMS AND CONDITIONS

Sep 29, 2014

This is a License Agreement between Fei Yang ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard, Langford Lane Kidlington, Oxford, OX5 1GB, UK
Registered Company Number	1982084
Customer name	Fei Yang
Customer address	404 Life Science Building, BATON ROUGE, LA 70803
License number	3478400557830
License date	Sep 29, 2014
Licensed content publisher	Elsevier
Licensed content publication	Crop Protection
Licensed content title	Susceptibility of Louisiana and Florida populations of Spodoptera frugiperda (Lepidoptera: Noctuidae) to transgenic Agrisure®Viptera™ 3111 corn
Licensed content author	Fei Yang, Fangneng Huang, Jawwad A. Qureshi, B. Rogers Leonard,Ying Niu, Liping Zhang, David S. Wangila
Licensed content date	August 2013
Licensed content volume number	50

Licensed content issue number   n/a

Number of pages   3

Start Page   37

End Page   39

Type of Use   reuse in a thesis/dissertation

Portion   full article

Format   both print and electronic

Are you the author of this Elsevier article?   Yes

Will you be translating?   No

Title of your thesis/dissertation   EVALUATION OF PYRAMIDED BT CORN FOR
                                    MANAGEMENT OF CORN EARWORM AND FALL
                                    ARMYWORM (LEPIDOPTERA: NOCTUIDAE)

Expected completion date   Oct 2014

Estimated size (number of pages)   150

Elsevier VAT number   GB 494 6272 12

Permissions price   0.00 USD

VAT/Local Sales Tax   0.00 USD / 0.00 GBP

Total   0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time athttp://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions.   These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction.   In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you.   Notice of such denial will be made using the contact information provided by you.   Failure to receive such notice will not alter or invalidate the denial.   In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world**English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must

maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

For journal authors: the following clauses are applicable in addition to the above: Permission granted is limited to the author accepted manuscript version* of your paper.

*Accepted Author Manuscript (AAM) Definition: An accepted author manuscript (AAM) is the author's version of the manuscript of an article that has been accepted for publication and which may include any author-incorporated changes suggested through the processes of submission processing, peer review, and editor-author communications. AAMs do not include other publisher value-added contributions such as copy-editing, formatting, technical enhancements and (if relevant) pagination.

You are not allowed to download and post the published journal article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version. A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Posting to a repository: Authors may post their AAM immediately to their employer's institutional repository for internal use only and may make their manuscript publically available after the journal-specific embargo period has ended.

Please also refer to Elsevier's Article Posting Policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only.. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the

printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Elsevier Open Access Terms and Conditions

Elsevier publishes Open Access articles in both its Open Access journals and via its Open Access articles option in subscription journals.

Authors publishing in an Open Access journal or who choose to make their article Open Access in an Elsevier subscription journal select one of the following Creative Commons user licenses, which define how a reader may reuse their work: Creative Commons Attribution License (CC BY), Creative Commons Attribution – Non Commercial - ShareAlike (CC BY NC SA) and Creative Commons Attribution – Non Commercial – No Derivatives (CC BY NC ND)

Terms & Conditions applicable to all Elsevier Open Access articles:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation.

The author(s) must be appropriately credited.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: You may distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text or data mine the article, including for commercial purposes without permission from Elsevier

CC BY NC SA: For non-commercial purposes you may distribute and copy the article, create extracts, abstracts and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text and data mine the article and license new adaptations or creations under identical terms without permission from Elsevier

CC BY NC ND: For non-commercial purposes you may distribute and copy the article and include it in a collective work (such as an anthology), provided you do not alter or modify the article, without permission from Elsevier

Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

· Promotional purposes (advertising or marketing)

· Commercial exploitation (e.g. a product for sale or loan)

· Systematic distribution (for a fee or free of charge)

Please refer to Elsevier's Open Access Policy for further information.

21. **Other Conditions**:

v1.6

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

A.3. Letter of Permission for Chapter 4

<div align="center">

ELSEVIER LICENSE TERMS AND CONDITIONS

</div>

Sep 29, 2014

This is a License Agreement between Fei Yang ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard, Langford Lane Kidlington, Oxford, OX5 1GB, UK
Registered Company Number	1982084
Customer name	Fei Yang
Customer address	404 Life Science Building, BATON ROUGE, LA 70803
License number	3478410671827
License date	Sep 29, 2014
Licensed content publisher	Elsevier
Licensed content publication	Crop Protection
Licensed content title	Occurrence, distribution, and ear damage of *Helicoverpa zea* (Lepidoptera: Noctuidae) in mixed plantings of non-Bt and Bt corn containing Genuity® SmartStax™ traits
Licensed content author	Fei Yang, David L. Kerns, Graham P. Head, B. Rogers Leonard, Ying Niu, Fangneng Huang
Licensed content date	January 2014
Licensed content volume number	55

Licensed content issue number   n/a

Number of pages   6

Start Page   127

End Page   132

Type of Use   reuse in a thesis/dissertation

Portion   full article

Format   both print and electronic

Are you the author of this Elsevier article?   Yes

Will you be translating?   No

Title of your thesis/dissertation   EVALUATION OF PYRAMIDED BT CORN FOR MANAGEMENT OF CORN EARWORM AND FALL ARMYWORM (LEPIDOPTERA: NOCTUIDAE)

Expected completion date   Oct 2014

Estimated size (number of pages)   150

Elsevier VAT number   GB 494 6272 12

Permissions price   0.00 USD

VAT/Local Sales Tax   0.00 USD / 0.00 GBP

Total   0.00 USD

Terms and Conditions

INTRODUCTION

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions.   These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction.   In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you.   Notice of such denial will be made using the contact information provided by you.   Failure to receive such notice will not alter or invalidate the denial.   In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world**English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must

maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing athttp://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

For journal authors: the following clauses are applicable in addition to the above: Permission granted is limited to the author accepted manuscript version* of your paper.

*Accepted Author Manuscript (AAM) Definition: An accepted author manuscript (AAM) is the author's version of the manuscript of an article that has been accepted for publication and which may include any author-incorporated changes suggested through the processes of submission processing, peer review, and editor-author communications. AAMs do not include other publisher value-added contributions such as copy-editing, formatting, technical enhancements and (if relevant) pagination.

You are not allowed to download and post the published journal article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version. A hyper-text must be included to the Homepage of the journal from which you are licensing athttp://www.sciencedirect.com/science/journal/xxxxx. As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Posting to a repository: Authors may post their AAM immediately to their employer's institutional repository for internal use only and may make their manuscript publically available after the journal-specific embargo period has ended.

Please also refer to Elsevier's Article Posting Policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only.. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the

printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Elsevier Open Access Terms and Conditions

Elsevier publishes Open Access articles in both its Open Access journals and via its Open Access articles option in subscription journals.

Authors publishing in an Open Access journal or who choose to make their article Open Access in an Elsevier subscription journal select one of the following Creative Commons user licenses, which define how a reader may reuse their work: Creative Commons Attribution License (CC BY), Creative Commons Attribution – Non Commercial - ShareAlike (CC BY NC SA) and Creative Commons Attribution – Non Commercial – No Derivatives (CC BY NC ND)

Terms & Conditions applicable to all Elsevier Open Access articles:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation.

The author(s) must be appropriately credited.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: You may distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text or data mine the article, including for commercial purposes without permission from Elsevier

CC BY NC SA: For non-commercial purposes you may distribute and copy the article, create extracts, abstracts and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text and data mine the article and license new adaptations or creations under identical terms without permission from Elsevier

21. **Other Conditions**:

v1.6

VITA

Fei Yang was born in Jurong, Jiangsu Province, China. After graduating from high school, he attended Yangzhou University in Yangzhou, China where he received the degree of Bachelor of Science in Ecology in 2008. After graduation, Fei enrolled into the graduate program in the department of Horticultural and Plant Protection at Yangzhou University from 2008 to 2011, where he received the degree of Master of Science in Agricultural Insect and Pest Control. His master's research focused on the survey of the distribution of *Liriomyza* species in Jiangsu and analysis of mitochondrial genomes of three *Liriomyza* species.

In May, 2011, Fei began his doctoral studies under Dr. Fangneng Huang in the Department of Entomology at Louisiana State University. Currently, he is a doctoral candidate in the Department of Entomology. His dissertation research has focused on evaluation of pyramided Bt corn for management of corn earworm and fall armyworm (Lepidoptera: Noctuidae). He is currently completing the requirements for the degree of Doctor of Philosophy and plans to pursue his career in insect pest management research.

Fei was married to Xuan Chen and they have a lovely son, Louis Yang.