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Larval Survival and Movement of the Fall Armyworm and Corn Earworm (Lepidoptera: Noctuidae) in Seed Blends of Non-Bt and Pyramided Bt Corn: Implications for Resistance Management

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LARVAL SURVIVAL AND MOVEMENT OF THE FALL ARMYWORM AND CORN EARWORM (LEPIDOPTERA: NOCTUIDAE) IN SEED BLENDS OF NON-BT AND PYRAMIDED BT CORN: IMPLICATIONS FOR RESISTANCE MANAGEMENT

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ABSTRACT

A seed blend refuge has been used in the U.S. Corn Belt for Bt corn insect resistance management (IRM). The fall armyworm, *Spodoptera frugiperda* (F.), and corn earworm, *Helicoverpa zea* (Boddie) are major target pests of Bt corn in the Americas. One major concern for the use of a seed blend refuge is that larval movement of insects among non-Bt and Bt plants could affect its efficacy for resistance management. To address this concern, field and greenhouse trials were conducted to determine 1) if seed blend refugia could provide comparable levels of susceptible *S. frugiperda* (aabb) as structured refugia and 2) if seed blends created a more favorable environment for a *S. frugiperda* Cry1A.105/Cry2Ab2-dual-gene heterozygous genotype (AaBb) over aabb. Live larvae and plant injury were virtually not observed on Bt plants across all planting patterns. Performance of aabb on non-Bt plants was similar between seed blends and pure non-Bt plantings, suggesting that blend refugia might be able to provide an equivalent susceptible population of *S. frugiperda* as structured refugia. In the greenhouse, two insect genotypes, a homozygous susceptible (aabb) and a dual-gene heterozygous (AaBb), on seed blends performed similarly, indicating that seed blends did not create more favorable conditions for AaBb over aabb in the test condition. Additionally, three caged-field trials were conducted to evaluate larval movement and survival of *H. zea*, in eight seed blends of Cry1Ab/Vip3A corn with 0-30% non-Bt refuge. No live larvae or kernel damages were observed on all Bt plants across all trials and planting patterns. In pure non-Bt plantings, 64.8% larvae moved away from infested ears and survived on other plants, but most larvae (86.8%) located within a distance of three plants from the central plants, and all larvae were found on the central or its adjacent rows. Larval survival and distribution were similar among seed blends with non-Bt plants in the centers, in which larvae were predominately (85.7%) located on the central

plants and 100% were found on the central rows. Overall, larval recovery from central non-Bt plants in seed blends was 27.5% less than that from pure non-Bt plantings. Kernel damage levels followed similar patterns as larval survival, and recovered larvae developed similarly across treatments. The results suggest that *H. zea* larvae moved significantly in corn fields, and such movement can reduce susceptible insect populations hosted in seed blend refuge. Data generated from this thesis should have value in understanding larval movement behavior of *H. zea* in corn fields, as well as managing *S. frugiperda* and *H. zea* and evaluating if send blend refugia could be a suitable method for Bt crop resistance management.

1. INTRODUCTION

1.1. Corn production in the U.S.

Corn (*Zea mays*) is a major crop in the U.S, and the cultivation of corn has an important role in the national economy. In 2019, corn was planted on a total of 91.7 million acres in the U.S., up three percent from the previous season (NASS, 2019). In Louisiana, corn is also a major field crop. The total area planted in 2019 was estimated at 570,000 acres, up 24% from 2018 (NASS, 2019). The U.S. Department of Agriculture (USDA) reported that, in 2019, approximately 90% of the total corn area planted was comprised of genetically engineered varieties. Bt corn acreage in the U.S. increased from nearly 8% in 1997 to 19% in 2000, before climbing to 83% in 2019 (USDA, 2019a; 2019b).

The year 1996 marked the first year in which an area of approximately 1.7 million hectares of crops containing GM traits was planted (Brookes, 2014). Since then, a considerable increase in the adoption of GM crops has occurred; by 2018, the area of genetically modified (GM) crops planted globally reached 191.7 million hectares (or 473.7 million acres), an increase of approximately 113-fold (ISAAA, 2018). Because of this, GM crops are considered the most rapidly adopted crop technology in the history of modern agriculture. Since this technology was first commercialized, the U.S. has been one of the leading countries in the usage of insecticidal *Bacillus thuringiensis* (Bt) crops, as well as the overall GM crops. Globally, the main biotech crops are soybeans, corn, cotton, and canola (ISAAA, 2018). Additionally, Bt sugarcane has been recently planted in Brazil. Today, insecticide resistance (Bt crop technology), along with herbicide resistance, has been one of the most used biotech traits in agriculture. The environmental safety characteristics, high efficiency against target insect-pests, and reduced needs of insecticide usage have enhanced the rapid adoption of the Bt crop technology.

1.2. Corn usage in the U.S.

Corn is one of the most important crops worldwide. Due to its nutritive value, richness in fibers, proteins, carbohydrates, and vitamins, corn has been a staple of American agriculture for decades. Corn is an adaptable crop that grows in diverse climates and soil. Most of the corn harvested in the U.S. is utilized for a vast array of products. In 2019, 34.9% of the corn was used as food and food-related products, seed, and for industrial purposes; 27.5% was utilized as ethanol and ethanol by-products; 27% became residual and feed for animal meat such as beef, poultry, and swine; and the last portion (10.6%) was destined for exportation (USDA, 2019a).

1.3. Major insect pests of corn

Corn is highly attractive to many insect pests. Every part of the plant has the potential to become a host for herbivorous insects. The insect pests of corn are numerous, but the most important can be divided into four major groups: seed, root, and lower stem feeders; stalk borers; leaf feeders; and ear feeders (Radcliffe and Hutchison, 1999).

Wireworms and seed corn maggots are the most common pests of seedling corn. In addition, wireworms and white grubs can feed on corn roots during cropping seasons and may be found in the soil during the year. Rootworms such as western corn rootworm (*Diabrotica virgifera virgifera*) and southern corn rootworm (*Diabrotica undecimpunctata howardi*) attack the corn root system. Furthermore, billbugs, chinch bugs (*Blissus leucopterus leucopterus*), and black cutworms (*Agrotis ipsilon*) are known to attack the lowest parts of the stem. Another group of corn pests are stalk borers. The insects of this group bore into corn stalks but may also cause injury to leaves, ears, or other parts of the plant during their life cycle. These insects cause injuries that affect the plant vascular system, which can result in stunting and lodging as plants mature. The main insects in this group are composed by the European corn borer (*Ostrinia*

nubilalis), southwestern corn borer (*Diatraea grandiosella*), sugarcane borer (*Diatraea saccharalis*), southern cornstalk borer (*Diatraea crambidoides*), and lesser cornstalk borer (*Elasmopalpus lignosellus*). The third group of corn pests is the leaf feeders. Insects feeding on corn leaves are directly responsible for the reduction of plant photosynthesis. Aphids remove sap from the plant, while cutworms, armyworms, and beetles are responsible for leaf removal. Each of these feeding behaviors results in chlorophyll reduction, which negatively impacts photosynthesis. The last category of corn pests are composed by the ear feeders. Pest damage on corn ears directly affects yield and aesthetics, which can cause severe economic losses for growers. The main ear feeders are the corn earworm (*Helicoverpa zea*), fall armyworm (*Spodoptera frugiperda*), and western bean cutworm (*Striacosta albicosta*). Both S. *frugiperda* and *H. zea* are major insect pests in Louisiana and other areas throughout Southern U.S. (Siegfried et al., 2000; Buntin et al., 2004; Chilcutt et al., 2007; Siebert et al., 2012; Capinera, 2017a; 2017b).

1.4. Fall armyworm (S. *frugiperda*) – biology and distribution

S. *frugiperda* is a pest of a wide range of host plants. Known to be a long-distance migratory insect, adults have highly mobile characteristics, being distributed throughout the USA (Luginbill 1928; Sparks, 1979). S. *frugiperda* is a multivoltine species that has a life cycle characterized by eggs, larvae with normally five or six instars, pupa, and adult moths. Six or more generations per season may survive and develop in zones of the Southern U.S. (Luginbill, 1928). Female moths lay their eggs organized in egg masses, and an egg mass usually contains 100 to 200 individual eggs per oviposition (Pitre et al., 1983). Moreover, a moth can oviposit over 1,000 eggs during its lifetime (Luginbill, 1928). Neonates tend to consume the residual of the egg masses where they hatch from after eclosion. Second instar larvae then scatter around

host plants to feed on leaf tissue. *S. frugiperda* larvae are highly mobile, and neonates hatched from an egg mass can disperse to many adjacent plants. The behavior of *S. frugiperda* is complicated to predict because there are two distinct, but morphologically similar strains (Hardke et al., 2015). These two strains are known as "host specific" in reference to the preference for a specific host plant. The common names of these two strains are corn-strain (C-strain) and rice-strain (R-strain) (Quisenberry, 1991; Nagoshi and Meagher, 2004). C-strain and R-strain can be differentiated by genetic markers (Nagoshi and Meagher, 2004**).** Integrated pest management (IPM) approaches can differ significantly because C-strain and R-strain present variations in the life cycle (Hardke et al., 2015). The two *S. frugiperda* strains may show distinct behaviors that can alter control tactics (Veenstra, 1994; Nagoshi and Meagher, 2004). The differences include mating, larval development, insecticide resistance, plant tissue use, and susceptibility to Bt crops (Veenstra, 1994; Nagoshi and Meagher, 2004). Due to the long-distance migration capacity and generalist feeding behavior of *S. frugiperda*, alternative tactics to manage this pest became less efficient. Before the advent of Bt crops, *S. frugiperda* control tactics were heavily dependent on insecticides (Young, 1979). Consequently, this insect is resistant to many classes of insecticides worldwide (Yu, 1992; Berta et al., 2000; Pitre, 1988; Belay, 2012; Gutiérrez-Moreno et al., 2018).

1.5. Corn earworm (*H. zea*) – biology and distribution

   H. zea is considered to be the most costly crop pest in North America (Capinera, 2017a). This species has other common names, but only two are recognized by the Entomological Society of America: cotton bollworm and tomato fruit worm. *H. zea* is a polyphagous pest that has more than 200 host plants, many of which are economically important crops in the Americas, such as corn, cotton, grain sorghum, and soybean (Lingren et al., 1994). A mated female moth

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usually deposits between 500 and 3000 pale green eggs during its life cycle. Eggs are first yellowish, and posteriorly become gray in about three to four days (Neunzig, 1964). Larvae have five to six instars. Damage to corn is primarily a result of larvae feeding on ear kernels. Last-instar larvae pupate below the soil surface. Critical factors for pupation are temperature and soil moisture. Low temperature and high soil moisture can increase mortality of pupae (Barber, 1937; Ditman et al., 1940). Caprio and Benedict (1996) considered soil moisture, temperature, and cultivation practices as important factors related to overwintering survival of *H. zea* (Caprio and Benedict, 1996). Insecticide resistance management (IRM) programs must look at overwintering carefully because resistant insects must overcome winter temperatures to reproduce. *H. zea* are usually not able to overwinter in the northern states of the U.S., but there are concerns that selection pressure and resistance development might increase in southern states where populations are potentially exposed to both transgenic cotton and transgenic corn. *H. zea* moths live on average 7.5 to18 d. Adults feed on the nectar of several plants (Nuttycombe, 1930). Previous studies documented patterns of *H. zea* long-distance migration in North America (Fitt, 1989; Gould et al., 2002). In the Southern U.S. where farmers also grow cotton, *H. zea* moves to other hosts after corn senescence, particularly cotton, soybean, and grain sorghum, for two to three additional generations (US EPA, 2001).

1.6. Corn herbivory by *S. frugiperda* and *H. zea*

Although *S. frugiperda* larvae can feed on corn ears, it mainly feeds inside the whorl of the vegetative stage plants, and the injury can be easily observed (Luginbill, 1928). Neonates generally start eating one side of the foliage, leaving the other side intact. Later instar larvae feed towards inside from the edge of the leaves, making holes and galleries (Luginbill, 1928). The final instar consumes an amount higher than the sum of previous instars (Luginbill, 1928).

Marenco et al. (1992) showed that different whorl stages in sweet corn present different levels of tolerance to injury when attacked by *S. frugiperda*. For instance, earlier whorl stages were the most tolerant to injury, the mid-whorl stage intermediate, and the late whorl stage was the least tolerant. For instance, late whorl stages with mean densities of 0.2 to 0.8 larvae per plant had yield reduction of 5 to 20 percent, respectively (Marenco et al., 1992).

Sometimes, *S. frugiperda* larvae will reach the corn ear to feed on kernels in the same way as *H. zea*. However, *H. zea* larvae tend to initially feed on the silk, reaching the apical parts of the ear before moving down to the kernels. In contrast, *S. frugiperda* larvae reach kernels by creating holes on the lateral side of the ears through the husk. A previous study reported that corn leaf tissues of younger vegetative stages are more suitable for survival and development of *S. frugiperda* when compared to older vegetative stages (Pannuti et al., 2015). Moreover, corn leaves of older vegetative stage tend to be unsuitable for larval development, which may induce *S. frugiperda* larvae to feed on silk tissues and ear kernels (Pannuti et al., 2015). Although *S. frugiperda* larvae can exhibit this behavior, Pannuti et al. (2015) demonstrated that silk tissues are not suitable for *S. frugiperda* larval development. Additionally, larvae feeding on corn kernels were able to grow faster than larvae feeding on other parts of the plant (Pannuti et al., 2015).

Figure 1.1. Corn leaf injury caused by S. *frugiperd*a. Photo by Marcelo Dimase

Figure 1.2. *S. frugiperda* larva. Photo by Fangneng Huang

H. zea is considered a significant insect pest of agricultural crops in North America (Capinera,

2017a; DiFonzo and Porter, 2018). Larvae initially feed on corn silks, gaining access to the corn

ear from the tip (Wiseman et al. 1978). Larval development can occur by feeding on the silk and

ear tip only, or by damaging an extent of the corn ear as large as half of its size. Corn ear feeding

has been reported by several corn-producing states within the U.S. and appears to be the most

common type of damage caused by *H. zea* larvae (Bohnenblust et al., 2013; Reay-Jones et al.,

2016). Corn leaf injury can also be observed; however, this type of feeding is not common

(Quaintance and Brues, 1905). Besides damage on corn ears, *H. zea* injury may create an

entrance for mycotoxin colonization. Aflatoxins and fumonisins are two major mycotoxins that

impact the health of humans and animals (Widstrom, 1996, Munkvold, 2003). However, the

correlation of corn ear damage with concentrations of aflatoxins and fumonisins is not strong

(Bibb et al. 2018).

Figure 1.3. H. *zea* larva feeding or corn ear.
Photo by Marcelo Dimase.

1.7. Non-transgenic control tactics

1.7.1. Biological control

S. frugiperda survival and development are favored by cool weather and periods of rainfall (Luginbill, 1928). Such environmental conditions allow *S. frugiperda* to reproduce successfully and migrate north before the proliferation of natural enemies that keep *S. frugiperda* in check (Luginbill, 1928). Luginbill (1928) grouped the natural enemies of *S. frugiperda* in three categories: invertebrates, vertebrates, and diseases (Luginbill, 1928). Invertebrates are both parasitic and predacious enemies (Luginbill, 1928). Several parasitoids that are known to affect *S. frugiperda* include insects that belong to the orders Diptera and Hymenoptera. For instance, *Archytas marmoratus* (Townsend) (Diptera: Tachinidae) are fly parasitoids usually associated with *S. frugiperda* and other noctuid species in South America (Guimarães, 1977; Virla et al., 1999). Additionally, wasp parasitoids are commonly reported emerging from larvae in sweet corn in South Florida (Meagher et al., 2016). However, occurrence and dominance of parasitoids may vary in different locations and throughout the year. Furthermore, the species that attack *S. frugiperda* are usually generalist predators of other caterpillars. Examples of important insect predators of *S. frugiperda* such as earwigs, ground beetles, spined soldier bug, *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae), and flower bug, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae). In addition, a variety of vertebrates such as rodents, skunks, and birds also utilize *S. frugiperda* larvae as food sources. Finally, several pathogens, including fungi, protozoa, bacteria, viruses, and nematodes have been linked to *S. frugiperda* mortality (Gardner et al. 1984). Although fungal and viral pathogens have the potential to cause elevated levels of mortality in *S. frugiperda* populations, disease generally appears after *S. frugiperda* already caused economic injury (Gardner et al., 1984).

H. zea larvae often find greater protection from predators or parasitoids inside corn ears compared to soybean and cotton (Manley et al., 1991). Manley et al. (1991) documented that approximately 6% of *H. zea* larvae recovered from ears were parasitized in experiments conducted in South Carolina. The most common parasitoid collected was *Archytas marmoratus* (Townsend) (Diptera: Tachinidae) (Manley et al., 1991). *A. marmoratus* inundative releases in Georgia demonstrated the possibility of becoming part of IPM programs (Proshold et al. 1998). . Invertebrate predators of *H. zea* include, but are not limited to *Orius insidiosis* (Say) (Reid, 1991) and *Harpalus pennsylvanicus* DeGeer (Lesiewicz et al. 1982). Vertebrate predators include a range of birds such as the red winged blackbirds (Mott and Stone, 1973), skunks, and rodents. Finally, nematodes have been reported as potential natural enemies of *H. zea*. In addition, viruses such as the nucleopolyhedroviruses (NPV) can be used as biopesticides to reduce pest populations (Pingel and Lewis, 1999). Although a range of natural enemies can provide some level of control of *H. zea*, they are not often effective enough to reduce *H. zea* populations to prevent or to minimize damage. Overall, the impact of pathogens generally occurs after the insect has already caused significant damage to the corn ears.

1.7.2. Cultural control

Luginbill (1928) suggested that *S. frugiperda* injury could be reduced if grass was managed in southern U.S. fields. Since field infestations start on grass, cleaning the edges of fields could prevent *S. frugiperda* progression from grasses to crops (Luginbill, 1928). In the southern U.S., early planting in addition to the use of varieties that mature earlier are cultural practices widely adopted by corn producers. Also, harvesting corn earlier is a cultural strategy that allows the corn ears to be collected before *S. frugiperda* density increases in the field (Mitchell, 1978). In

addition, reduced tillage appears to have an insignificant impact on the management of *S. frugiperda* populations (All, 1988).

A limited number of studies have been conducted to investigate the impact of cultural practices to manage *H. zea*. Early plating can reduce the pressure of *H. zea* at later stages during the season (Buntin et al., 2004). Moreover, corn has been implemented as a trap crop for *H. zea* in association with other crops such as cotton (Lincoln and Isely, 1947) and soybeans (Javaid et al., 2005). Furthermore, increasing rates of nitrogen-based fertilizers are associated with higher husk tightness and decreased adult pressure at the R1 stage, which are factors that ultimately help to reduce *H. zea* infestations (Klostermeyer, 1950). Additionally, tillage has been used in the past as a control tactic to reduce *H. zea* populations (Barber and Dicke, 1937), due to the behavior of *H. zea* to pupate in the soil during overwintering (Phillips and Barber, 1929). Tillage functions by disrupting tunnels from which larvae emerge or by leading to death directly (Barber and Dicke, 1937). However, due to the wide range of *H. zea* host plants in the southern areas of the U.S., tillage may not be a suitable cultural practice to decrease *H. zea* populations (Montgomery, 2007).

1.7.3. Host plant resistance

Wiseman and Davis (1990) classified antibiosis, non-preference (later named antixenosis), and tolerance as mechanisms of plant resistance in corn against *H. zea*. However, these terms are best described as "types" or "categories" of plant resistance (Smith et al., 2005). Overall, the information available about types of plant resistance against *S. frugiperda* is extremely limited in the literature. Scott et al. (1977) released the first known corn germplasm to have resistance against *S. frugiperda*. Wiseman et al. (1981) related a smaller larval weight found on resistant hybrids of corn as evidence of antibiosis. Furthermore, a choice test demonstrated that *S.*

frugiperda larvae consistently preferred to feed on susceptible varieties (Wiseman et al., 1981). For this reason, both antibiosis and antixenosis were hypothesized to be resistance traits in that particular corn genotype (Wiseman et al., 1981); additionally; similar observations were documented in further studies (Williams et al., 1983; 1985; 1989; Wiseman et al., 1983; Videla et al., 1992).

Several plant traits – husk tightness, shorter husk length, and higher quantity of moisture in longer and tighter silks due to larger silk channels - have been associated with reductions in levels of *H. zea* larvae damage because of the lower rates of larvae reaching the corn ears (Wiseman et al., 1977; Rector et al., 2002; Ni et al., 2007). Antibiosis impact on *H. zea* might change significantly across different corn hybrids (Knapp et al., 1967; Wiseman and Snook, 1996). Maysin, a glycosyl flavone, reduced *H. zea* larval development in corn as a result of antibiosis resistance (Waiss et al., 1979). Antixenosis was documented to oviposition (Widstrom et al., 1979) and silk feeding in some genotypes of corn (Wiseman et al., 1983).

1.7.4. Chemical control

S. frugiperda may cause up to 45% yield losses in corn without control measurements (Hruska and Gladstone, 1988). In some tropical environments, corn fields can be completely lost if *S. frugiperda* is not controlled (Blanco et al., 2016). Before the advent of Bt crops, farmers relied on synthetic insecticides as the main strategy to control *S. frugiperda* populations (Young, 1979; Yu, 1991; 1992; Al-Sarar et al., 2006; Blanco et al., 2010). Since the first year genetically modified crops expressing Bt proteins became available, the use of insecticides has been reduced by 47.8% in the U.S. (Brookes and Barfoot, 2017). Nevertheless, insecticides continue to be highly adopted in countries where Bt crops are not commercialized (Gutiérrez-Moreno et al., 2018). In the southern areas of the U.S., synthetic insecticides are mostly used in sweet corn

fields to control *S. frugiperda*. Additionally, insecticides can also be applied through overhead irrigation systems. Granular formualtions of insectcides applied to the whorl of the plant can be an alternative method of control (Young, 1979). Reduced applications of insecticides during the silking stage can be achieved if plants are maintained free of larvae at younger vegetative stages (Foster, 1989).

 H. zea control with foliar insecticides is difficult due to the movement of this pest through silking channels and protection found in the surrounding ears (Widstrom et al., 1976). Reay-Jones and Reisig (2014) reported that *H. zea* damage to kernels continued despite intensive applications (13-18 per trial) of chlorantraniliprole. Likewise, chlorantraniliprole or flubendiamide applications in Georgia and Mississippi corn fields between the emergence of silks and its senescence did not reduce kernel damage significantly in treated plots of Bt corn expressing Cry1F protein (Bibb et al., 2018). The application of insecticides in large amounts is costly and restrictive for corn growers attempting to reduce losses caused by *H. zea* (Reay Jones et al., 2019). Conversely, crops designated to fresh markets such as sweet corn have low tolerance to *H. zea* damage (Shelton et al., 2013). Furthermore, insecticide applications are a critical factor because short intervals of application during silk emergence are required to impede *H. zea* from progressing to the ear (Shelton et al., 2013).

1.8. Transgenic corn: *Bacillus thuringiensis* (Bt)

 Bacillus thuringiensis (Bt) is a gram positive, entomopathogenic bacterium that forms spores; moreover, Bt bacteria are aerobic and usually established in soil, dead insects, and locations that contain water (Lambert and Peferoen, 1992). Bt produces a crystalline (Cry) protein with insecticide activity during the sporulation phase (Jouzani et al., 2008a; 2008b). This species of bacteria is known as the most efficient bio-insecticide currently, being capable of transmitting

toxicity to a wide range of insect orders such as Diptera, Coleoptera, and Lepidoptera (Federici et al., 2006; Lacey et al., 2015). Bt has been successfully used as a bio-insecticide with nearly 100 formulated sprayable pesticides (Lacey et al. 2015). Furthermore, Bt features include host-specificity and fast-acting properties, which limits negative effects against non-target organisms (Lacey et al. 2015; Jain et al. 2016). On the global transgenic Bt crop market (corn, cotton, and soybean), Bt genes targeting lepidopteran pests can be divided into three categories (Yang et al., 2016): 1) Cry1 group: Cry1Ab, Cry1Ac, Cry1F, and Cry1A.105; 2) Cry2 group: Cry2Ab and Cry2Ae; and 3) Vip3A, which is a vegetative insecticidal Bt protein (Estruch et al., 1996; Warren, 1997; Yu et al., 1997). The groups of crystalline proteins (Cry), also known as δ-endotoxins, are formed during the sporulation phase of the bacterium, and display toxicity due to the synthesis of Cry proteins that are converted into toxic peptides in the midgut by a series of events that happen after larvae ingest Bt, causing cell disruption and death of susceptible insects (Schnepf et al., 1998; Bravo et al., 2007). Vip3A represents a novel mode of action compared to other Cry proteins (Estruch et al., 1996; Lee et al., 2003; 2006; Kurtz, 2010), and shares no sequence homology with the known Cry proteins (Estruch et al., 1996). The Vip3A mode of action can be similar to some δ-endotoxin such as Cry1Ab, but Vip3A proteins do not use the same molecular targets and form different ion channels in comparison to Cry1Ab (Lee et al., 2003). Previous studies have shown that Vip3A proteins have a broad insecticidal spectrum, including toxic activity against many lepidopteran and coleopteran pests (Estruch et al., 1996; Warren, 1997; Yu et al., 1997). Vip3A proteins bind with receptors in the epithelial cells in the midgut of insects, which causes degeneration of epithelial layers (Yu et al., 1997). Relative to Cry1 and Cry2, which have been used in commercial Bt crops for many years, Vip3A is comparatively new.

Bt corn has been divided into two generations (Buntin and Flanders 2012; Huang et al., 2012). The first generation of Bt corn hybrids expresses a single Bt mode of action for a target species. Single-gene Bt corn products primarily targeted stalk borers and confer little efficacy against *H.zea* and *S.frugiperda*. (Adamczyk and Mahaffey, 2008; Huang et al., 2011). In order to increase the target spectrum and postpone resistance development, a gene pyramiding strategy was developed for transgenic Bt corn containing at least two Bt proteins expressing distinct modes of action (Monsanto, 2012). The second generation of pyramided Bt corn hybrids provided greater efficacy against both *H. zea* and *S. frugiperda* (Burkness et al., 2010; Niu et al., 2013). Currently, both *H. zea* and *S. frugiperda* are considered major target species for pyramided Bt corn technology (DiFonzo and Porter, 2018).

1.9. Bt Resistance

Farmers quickly adopted the use of Bt crops because of its ability against major target pests (James, 2018). However, the widespread acceptance of Bt crops creates an increased pressure for resistance development by the target species, which could accelerate the evolution of insect resistance (Ives et al., 2011). In fact, many cases of insect resistance to Bt crops have been reported in recent years. The first laboratory-selected example of Bt resistance was reported in the house fly (*Musca domestica*) (Harvey and Howell, 1965). Field resistance to foliar Bt insecticides was first reported in the diamondback moth (*Plutella xylostella*), a primary pest of vegetables (Tabashnik et al., 1990). Similarly, the cabbage looper (*Trichoplusia ni)* also exhibited resistance to Bt insecticide application in greenhouse (Janmaat and Myers, 2003). Currently, more than 20 cases of field resistance to Bt corn and Bt cotton have been reported in several countries (Tabashnik and Carrière, 2019; Huang et al., 2020), which include the African stem borer (*Busseola fusca*) to Cry1Ab corn in South Africa (Van Rensburg, 2007); western corn

rootworm (*Diabrotica virgifera virgifera*) to Cry3Bb1 corn in the U.S. (Gassmann et al., 2011); *S. frugiperda* to Cry1F corn in Puerto Rico (Storer et al., 2010), Brazil (Farias et al., 2014), the southeastern region of the U.S. (Huang et al., 2014), and Argentina (Chandrasena et al., 2018) and *H. zea* to Cry1A/Cry2A corn and cotton in the U.S. (Dively et al., 2016, Reisig et al., 2018; Kaur et al., 2019; Yang et al., 2019a). The wide development of resistance has become a great threat to the long-term sustainability of Bt crops as a viable pest management tool (Matten et al., 2012; Tabashnik et al., 2013; Huang, 2020). As major target pests of Bt corn and Bt cotton in the southern region of the U.S., *H. zea* and *S. frugiperda* represent a significant challenge for resistance management in the region.

1.10. Resistance management for Bt crops

1.10.1. High dose/refuge strategy

To delay the development of resistance, a mandatory Insect Resistance Management (IRM) plan referred as the "high dose/refuge" (HDR) strategy has been implemented since the first year Bt crops were planted in the U.S. (Matten et al., 2012). This strategy involves planting high-dose Bt corn that can kill resistant heterozygotes of the target species, while the remaining area is reserved for planting non-Bt varieties to serve as a refuge. High-dose Bt corn represents a concentration capable of killing at least 95% of a resistant heterozygous population of a target species (US EPA, 2001). The HDR strategy relies on the assumption that rare resistant insects can randomly mate with susceptible insects. Furthermore, most of the offspring carrying resistance alleles should subsequently be heterozygous and, therefore, be killed by 'high-dose' Bt corn plants. The HDR strategy has been used effectively in North America to provide resistance management for several important lepidopteran pests of both corn and cotton expressing Bt proteins (Huang et al., 2011). The planting requirements for structured refuge planting of Bt corn

traits targeting above-ground lepidopteran pests in the United States, outside the cotton-producing regions before 2010, were 20% (for single-gene expressed Bt corn) or 5% (for pyramided Bt corn) non-Bt refuge. On the other hand, the IRM plan requires growers to use a rate of 50% refuge for single-gene Bt corn or 20% refuge for pyramided Bt corn in the southern region where cotton is also planted (Matten et al., 2012).

1.10.2. Concerns involving the use of seed blends

Before 2010, only the "structured refuge" strategy was approved for use in Bt crop IRM. Beginning in that year, the U.S. EPA has conditionally approved a seed blend refuge strategy, also known as refuge-in-the-bag or RIB, as an alternative to planting pyramided Bt corn within the northern U.S. corn belt (US EPA, 2010a; 2010b; 2010c; Matten et al., 2012). In seed blends, a certain percentage of corn seeds expressing Bt proteins is mixed with a certain rate of corn seeds not expressing Bt traits. Thus, this strategy "forces" growers to purchase the premixed seeds for planting (Matten et al., 2012). Therefore, growers' compliance with the refuge requirement will no longer be an issue (Smith and Smith, 2014). As mentioned above, the current rate used in seed mixture strategy in the U.S. North Corn Belt is 95% Bt corn seeds mixed with 5% non-Bt corn seeds (Matten et al., 2012). However, scientific data supporting the seed blend strategy are still quite limited for the southern region of the U.S. (US EPA 2010a; 2010b; 2010c; 2018). Major concerns with seed blend usage include several aspects. First, larval movement of the target insect pests between Bt and non-Bt plants. In structural refuge, the movement behavior of adults is critical (Gould, 1994; Ostlie et al., 1997; Hellmich et al., 1999; Shelton et al., 2000; Qureshi et al., 2006), but with the RIB strategy, the main concern is that larval movement among Bt and non-Bt plants can contribute to the evolution of resistance in target pest populations (Wanglia et al., 2013). For instance, the movement of susceptible larvae from non-Bt refuge

plants to Bt plants in a RIB field could result in prominent mortality of susceptible insects compared to a structured refuge planting, resulting in decreased levels of susceptible individuals (Davis and Onstad., 2000). Second, some corn kernels may express no or low levels of Bt proteins, depending on the source of pollen fertilizing a kernel, because of the F2 segregation Consequently, differential susceptibility among instars (Huang 1999, 2006; Walker et al., 2000) and larval movement among Bt and non-Bt plants could create sublethal exposure and promote the build-up of resistance in target pest populations by increasing the survival of the resistant heterozygous or individuals carrying minor resistance alleles (Wangila et al., 2013). Third, some kernels of refuge plants may also express a low level of Bt proteins due to cross-pollination of the mixed plantings of Bt and non-Bt corn, which may kill the susceptible refuge insects, in particular for the ear feeding insects, such as *H. zea* (Yang et al., 2014a). A previous study demonstrated that cross-pollination of a commonly used pyramided Bt corn trait in 5:95% RIB planting caused more than 90% of refuge kernels to express more than one Bt protein (Yang et al., 2014a). For these reasons, the RIB was not considered to be a suitable IRM strategy for single-gene Bt corn (Yang et al., 2014a). Finally, similar Bt proteins are expressed in both Bt corn and Bt cotton and some targets are major pests for both crops (Yang et al., 2016). In the U.S. Cotton Belt where both Bt corn and Bt cotton are planted, selection pressure and resistance development risk is great. Similar challenges can also be present in other mixed cropping systems such as in Brazil where *S. frugiperda* is a cross-crop target of Bt corn, cotton, and soybean (Fitt, 2006; Huang et al., 2014; Sorgatto et al., 2015). The term cross-crop target refers to a resistant insect population selected with one Bt crop that is also resistant to other crops containing similar or different Bt genes (Yang et al., 2016). Several studies have documented that cross-resistance frequently occurs among closely-related Bt proteins and insect species

(Tabashnik et al., 2009; 2014; Brévault et al., 2013; Bernardi et al., 2015; Wei, 2015). However, no evidence of cross-resistance between Vip3A and Cry1 or Cry2 has been shown (Jackson et al., 2007; Sivasupramaniam et al., 2008; Niu et al., 2013; 2014; 2016; Vélez et al., 2013; Huang et al., 2014; Santos-Amaya et al., 2015; Yang et al., 2016; 2017). For this reason, the Vip3A gene has been incorporated into numerous Cry corn and Cry cotton products to provide pyramided Bt crops containing Cry1 or Cry2, as well as Vip3A. Some of these pyramided Bt corn and Bt cotton traits containing Vip3 and Cry1 or Cry2 have recently been commercialized, and many more are expected to become available. Nevertheless, the seed blend strategy has not been approved in the southern U.S., largely due to the lack of data to support (or disapprove) the strategy. Mathematical models (Carroll et al., 2012; Kang et al., 2012) have been established to evaluate IRM strategies for the planting of pyramided Bt corn, but due to the lack of the related scientific data for the modeling, those models did not incorporate these important factors mentioned above.

1.11. Considerations and research objectives

S. frugiperda is currently the only target-pest that has developed field resistance to Bt crops in several areas around the world. Recently, field resistance of *H. zea* to Bt corn and Bt cotton has also been widely reported in the U.S. Due to the broad Cry1F resistance in *S. frugiperda* (Huang et al., 2014; Bernardi et al., 2015; Santos-Amaya et al., 2015; Yang et al., 2016) and the surge of Cry1/Cry2 resistance in *H. zea* in the U.S. (Diverly et al., 2016; Tabashnik et al., 2017; Naik et al., 2018; Kaur et al., 2019), it is anticipated that the pyramided Bt crop traits containing the Vip3A gene will be widely planted in the U.S. and many other countries. In this context, an effective IRM strategy is essential to ensure the long-term effectiveness of the Vip3A protein against its major target insect pests. Although a few models have shown that seed blends could

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be an option to comply with IRM requirements to provide susceptible populations when planting

pyramid Bt corn (Carroll et al., 2012; Kang et al, 2012), published scientific information to

support the use of seed blends for pyramided Bt corn are lacking (Alyokhin, 2011; Onstad et al.,

2011; Gould and Anderson, 1991; Mallet and Porter, 1992; Davis and Onstad., 2000). If larval

movement is independent of the presence of Bt toxins, seed mixtures of Bt and non-Bt corn

could be an appropriate strategy to postpone the evolution of resistance to Bt crops (Mallet and

Porter, 1992). The ultimate goal of the present study was to provide necessary information to

assess whether seed blends could be an appropriate refuge strategy for Bt corn IRM in

controlling *S. frugiperda* and *H. zea*. The specific objectives of this research were to: 1)

determine the larval survival, development, and plant injury of susceptible *S. frugiperda* in

structure refuge and seed blends; 2) evaluate the performance of susceptible and dual-gene

heterozygous-resistant populations of *S. frugiperda* in structured refuge and seed bends; and 3)

understand larval movement of *H. zea* in structured and seed blend refuges, and analyze the

related effect on the supply of susceptible insect populations for IRM.

2. PERFORMANCE OF BT-SUSCEPTIBLE AND -HETEROZYGOUS DUAL-GENE RESISTANT GENOTYPES OF *SPODOPTERA FRUGIPERDA* (J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE) IN SEED BLENDS OF NON-BT AND PYRAMIDED BT CORN

2.1. Introduction

A mandatory insecticide resistance management (IRM) plan, named "high dose/refuge" (HDR) strategy, has been implemented since the first year of Bt crop adoption in the U.S. to delay resistance development (Matten et al., 2012). This strategy involves planting a portion of corn seeds with high dose Bt that will kill resistant heterozygotes of target pest, while the remaining seeds is cultured with non-Bt corn seeds to function as refugia for susceptible pest populations. There are two ways in U.S. North Corn Belt to plant the necessary non-Bt refuge corn for IRM: 1) structured refuge and 2) seed blend refuge. However, only the structured refuge is approved in the southern region of the U.S. where Bt cotton is also planted (Ostlie et al., 1997; Yang et al., 2015a).

Before 2010, 'structured refuge' was the only refuge method that was recommended in the U.S. for Bt corn IRM. For structured refugia, non-Bt corn is planted as blocks or strips with Bt corn (Ostlie et al., 1997). Global data analysis has shown that the HDR strategy with structured refugia is likely to be successful if its key assumptions are met (Huang et al., 2011). One problem is that the success of a structured refuge largely depends on grower's compliance. Several surveys have reported that grower's compliance with structured refugia in recent years has been deficient (Smith and Smith, 2014; US-EPA, 2018). Because of the compliance issue, the Environmental Protection Agency of the U. S. in 2010 conditionally allowed the seed blend method as another refuge option for the North Corn Belt of the U.S. where Bt cotton is not planted (US-EPA, 2018).

In seed blend refugia, a pre-determined rate of Bt and non-Bt seeds is blended in each seed sack before seeds are sold to growers. Therefore, grower's compliance with the structured refuge requirements will not be an issue (Smith and Smith, 2014; Yang et al., 2015a). The current seed blend rate used in the U.S. North Corn Belt is a blend of 5% non-Bt and 95% Bt seeds (Matten et al., 2012). Currently, seed blend refugia have not been used in the south region of the U.S. cotton region, largely due to the lack of necessary data to determine if seed blends are a suitable refuge method to be used in the region (Wanglia et al., 2013).

A major concern in using seed blend refugia is that larval dispersal of insects among non-Bt and Bt plants could negatively affect the efficacy of refuge blends (Mallet and Porter, 1992; Burkness et al., 2011; Razze and Mason, 2012). For example, the movement of susceptible larvae from non-Bt refuge plants to surrounding Bt plants in seed blends may result in greater mortality of the susceptible insects compared to the structured refuge planting (Davis and Onstad, 2000). In addition, heterozygous resistant individuals in seed blends may have advantages relative to their homozygous counterparts in seed blends. This is due to the dilution of Bt proteins when insects feed on non-Bt corn followed by feeding on Bt corn, and vice versa (Alyokhin, 2011; Onstad et al., 2011). A few provisional studies have compared the effect of seed blends for single- gene Bt resistance (Brévault et a1., 2015 ; Yang et al., 2016), but such potential advantage in seed blends has not been investigated for any heterozygotes that possess dual or multiple Bt resistance alleles.

In both North and South America, *S. frugiperda* is a major pest of field corn. Recently, this polyphagous pest has been introduced into Africa and Asia, and became a significant threat to food security of these regions (Goergen et al., 2016; Day et al., 2017; Wang et al., 2019). To date, *S. frugiperda* actually is the only major pest that has evolved practical resistance with field

control failures of Bt corn across multiple countries (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014; Omoto et al., 2016; Chandrasena et al., 2018). Recently, a dual-gene resistant strain of *S. frugiperda* that was highly resistant to corn plants containing pyramided Cry1A.105/Cry2Ab2 proteins (Niu et al., 2019; Zhu et al., 2019). The highly mobile behavior of *S. frugiperda* larvae plus the access to the dual-gene resistant insect strain offered us a good biological system to test the influence of seed blends on the performance of susceptible and resistant heterozygous genotypes. ln this study, field trials were conducted to investigate the effect of seed blends on the supply of susceptible *S. frugiperda* populations, while greenhouse tests were employed to assess if the performance of a susceptible and a dual-gene heterozygous-resistant genotypes in structured and seed blend refugia. Data generated from this study could be used to analyze if seed blend refugia are suitable for Bt corn IRM.

2.2. Materials and methods

2.2.1. Corn hybrids

Two pyramided Bt corn hybrids (DKC 66-87 and DKC 66-26), and one non-Bt corn hybrid (DKC 66-94) were provided by Bayer Crop Science (St. Louis, MO, IJSA). Hybrid DKC 66-26 possesses Trecepta® trait (Trecepta), while DKC 66-87 is a VT Double Pro trait (VT2P). Since 2010, VT2P has been commercially available, which expresses both Cry1A and Cry2A proteins targeting corn moth pests, including *S. frugiperda*. Trecepta is a relatively new Bt corn trait, which produces three Bt proteins, Vip3A, Cry2A and Cry1A targeting corn lepidopteran pests. The non-Bt hybrid, DKC 66-94, is genetically closely related to the two Bt corn hybrids.

2.2.2. Insect sources

Two insect genotypes, a susceptible (aabb) and a dual-gene Cry2A/1A resistant heterozygous (AaBb) genotype of *S. frugiperda* were used in the study. Genotype aabb was derived from two-

parental families collected from non-Bt corn fields near Winnsboro, Louisiana, in 2016. This genotype was susceptible to Cry1A, Cry2A, Cry1F, and Vip3A proteins, as well as to corn plants expressing one or more of these proteins (Yang et al., 2018; 2019a; Niu et al., 2019; Zhu et al., 2019). AaBb was generated from reciprocal hybrids between the susceptible genotype and a dual-gene Cry 2A/1A resistant genotype (AABB) of *S. frugiperda*, as described in Zhu et al. (2019). AABB was able to complete its life cycle on pyramided Cry 2A/1A Bt corn plants (e.g., VT2P) and the adults derived from the larvae feeding on VT2P plants produced normal progeny (Niu et al., 2019; Zhu et al., 2019).

2.2.3. Experimental design

This study consisted of three field trials and one greenhouse test. The field trials were designed to investigate larval survival, growth, and plant injury of genotype aabb in seed blends of non-Bt and Trecepta corn. The greenhouse study was designed to assess the performance of aabb and AaBb genotypes in seed blends of non-Bt and VT2P corn.

2.2.4. Field trials

Trial-I and Trial-II of the three field trials were conducted in 2018 and 2019, respectively, in East Baton Rouge Parish, USA, LA. Trial-III was conducted in 2019 in Rapides Parish, Louisiana, USA. Each trial contained six planting patterns of Trecepta and non-Bt corn with four rows (1 m row spacing) and 25 plants spaced ~15 cm apart in each row (4 x 25= 100 plants/plot). These six planting patterns were: 1) all non-Bt plants (Pure-nonBt), 2) all Bt plants (Pure-Bt), 3) a seed blend of 5% non-Bt and 95% Bt plants (5:95RIB); 4) a seed blend of 10% non-Bt and 90% Bt plants (10:90RlB) ; 5) a seed blend of 20% non-Bt and 80% Bt plants (20:80Rl B); and 6) a seed blend of 30% non-Bt plants and 70% Bt plants (30:70RIB). The 5:95% RIB is the currently adopted rate of the seed blend in the North Corn Belt of the U.S. for Bt corn IRM. The

pure non- Bt planting was for simulating the structured refuge planting as described in Ostlie et al. (1997). The other three refuge rates (10-30%) evaluated in this study are often discussed as possible seed blend rates for the U.S. southern region.

Microsoft Excel random number generator was used to determine the spots where non-Bt refuge seeds should be planted in each seed blend plot. At planting, two non-Bt seeds were hand-planted at each refuge location and marked with chopsticks, and 20% extra Bt seeds were hand-planted in each row to ensure optimum seed emergence. After plant emergence, plants were thinned to configure the designed plant densities/patterns. No insecticides were applied in the trial plots, while irrigation, fertilization, and herbicide application were used as needed. For each trial, treatment plots were arranged in a randomized complete block design (RCBD) with four replications. There was a 2-meter space between plots and 3-meter distance between blocks. Bt protein expression or non-expression was validated by testing leaf tissue using the EnviroLogix's ELISA kits (ME, USA).

In corn fields, *S. frugiperda* larvae mainly feed on plant leaf tissues during vegetative stages. To ensure an occurrence of sufficient insect populations for data collections, two (for Trial-I), three (Trial-II), or five (Trial-III) neonates of the aabb genotype were released on the leaves of every plant at V4-V5 plant stages, respectively. After manual infestations, larval survival was carefully monitored. When most live larvae on non-Bt corn plants in each trial reached 4th instars, larval survival, growth stage, and plant injury were checked for all plants (usually 9- 11 d after neonate release, depending on the weather conditions). Plant injury by *S. frugiperda* was measured using Davis' 1-9 rating scale, in which a rating of 1 refers no injury, while a rating of 9 means severe foliar injury (Davis et al., 1992).

2.2.5. Greenhouse tests

Greenhouse tests were employed to investigate the performance of AaBb and aabb individuals in five planting patterns of non-Bt and VT2P corn. Each planting pattern consisted of 4 rows and 14 plants in each row (56 plants per plot). The five planting patterns were 1) all non-Bt plants (Pure-nonBt), 2) all Bt plants (Pure-Bt), 3) a seed blend of 6 randomly planted non-Bt plants and 50 Bt plants (10:90RlB), 4) a seed blend of 11 randomly planted non-Bt plants and 45 Bt plants (20:80RlB), and 5) a seed blend of 17 randomly planted non-Bt plants and 39 Bt plants (30:70 RIB). The send blends of 6 non-Bt and 50 Bt plants, 11 non-Bt and 45 Bt plants, and 17 non-Bt and 39 Bt plants were designed to simulate 10:90% (non-Bt:Bt), 20:80%, and 30:70% seed blends, respectively. The five planting patterns were arranged in each of four greenhouse rooms in a RCBD with four replications, and greenhouse room was considered a block factor.

In the greenhouse study, four corn seeds were planted in 18.9-liter plastic pots containing Perfect Mix^{TM} soil (Expert Gardener products, St. Louis, MO), as described in Wanglia et al. (2013). The locations where non-Bt refuge seeds were planted in a RIB configuration were marked with chopsticks. After one week, plants were thinned to two plants per pot. Corn plants were 20 cm apart in a row and 60 cm from adjacent rows. There was 1 m between plots. Irrigation, fertilization, and hand weeding were used as needed to ensure optimum plant growth. As described in the field trials, expected Bt proteins in plants were validated with ELISA kits (EnviroLogix, ME, USA). When plants reached V4-V7 stages, three neonates (< 24 h old) of aabb or AaBb genotype were released on the leaves of each plant. Number of live larvae, larval growth, and plant injury ratings were recorded when most live larvae on non-Bt corn plants reached the 4th instars (usually after 10-13 d of neonate release) as described.

2.2.6. Data analysis

To facilitate data analysis, larval growth stages recorded in field trials and greenhouse tests were converted to larval growth index as described in Yang et al. (2014a). Also, data (e.g., number of live larvae per plant, larval growth index, and plant injury rating) recorded from non-Bt plants in seed blend plots were separated from those data observed from Bt plants as described in Wanglia et al. (2013). As reported in the results section of this chapter, virtually no live larvae survived, and little to no plant injury occurred on all Bt plants in both field trials and greenhouse tests across all planting patterns (Tables 2.1 and 2.2). Thus, data recorded from Bt plants were excluded in the analysis of variance (ANOVA). Data on number of live larvae per plant, larval growth index, and plant injury ratings observed from non-Bt plants were transformed to log $(x + 1)$ scale for normality and then subjected to one-way ANOVA with planting pattern as the main factor for each of the three open field trials or two-way ANOVA with planting pattern and insect genotype as the two main factors for the greenhouse tests (SAS Institute, 2010). Replication was included as a random effect in all models. ln addition, to raise degrees of freedom in the data analysis, data generated from the three field trials were pooled for each variable and the pooled data were also analyzed in mixed models with one-way ANOVA with trial as a random factor and planting pattern as the treatment factor (Kaur et al., 2019). Tukey's HSD tests at n = 0.05 were used to separate the treatment means. Untransformed data are presented in the tables.

2.3. Results

2.3.1. Larval survival of aabb in different planting patterns of non-Bt and Trecepta corn in open field trials

Across all three trials, only one live larva (4th instar) was found from Bt plants. Thus, data collected from Bt plants were not included in the statistical analysis. Analysis with data from only non-Bt plants showed that the effect of planting pattern on aabb larval survival was not

significant for each of the three trials ($F_{4,10\text{-}12} < 2.74$; $P > 0.0852$) or for the pooled data ($F_{4,41} = 1.34$; $P = 0.2717$) (Table 2.1). Based on pooled data, an average of 0.39 ± 0.11 (mean ± sem) larvae /plant was found in Pure-nonBt, and the number observed on non-Bt refuge plants in the three seed blends ranged from 0.26 ± 0.10 to 0.51 ± 0.13 (Table 2.1).

2.3.2. Larval growth index of aabb in different planting patterns of non-Bt and Trecepta corn in open field trials

The effect of planting patterns on larval growth index recorded from non-Bt plants was also not significant across each of the three trials ($F_{4,8\text{-}10} < 2.84$, $P > 0.0978$) and for pooled data ($F_{4,41} = 0.99$; $P = 0.4258$) (Table 2.1). The growth index of larvae recovered from Pure-nonBt was 3.72 ± 0.13 in pooled data, and the corresponding index for non-Bt refuge plants in the three seed blends ranged from 3.40 ± 0.28 to 3.76 ± 0.10 (Table 2.1).

2.3.3. Plant injury by aabb in different planting patterns of non-Bt and Trecepta corn in open field trials

Plant injury ratings in field trials were highly correlated to the larval occurrence described above. Little or no plant injury was observed on Bt plants across all trials and planting patterns with a plant injury rating of ≤ 1.28 in the pooled data (Table 2.1). Results from non-Bt plants showed that the effect of planting pattern on plant injury ratings was not significant in Trial-I ($F_{4,12} = 0.70$; $P = 0.6055$) and Trial-III ($F_{4,10} = 1.11$; $P = 0.4209$), but significant in Trial-II ($F_{4,11} = 7.70$; $P = 0.0033$) and for pooled data ($F_{4,41} = 2.94$; $P = 0.0317$) (Table 1). Based on pooled data, the injury rating (3.76 ± 0.59) observed on non-Bt refuge plants in 5:95RIB was significantly ($P < 0.05$) less than that (5.02 ± 0.45) observed in 20:80RIB, but not significantly different ($P > 0.05$) compared to those in pure-nonBt (4.65 ± 0.53) or the other two seed blends (4.18 ± 0.47 in 10:90RIB and 4.98 ± 0.51 30:70RIB) (Table 2.1).

Table 2.1. Larval survival, growth, and plant injury rating (mean ± sem) of *S. frugiperda* in seed blends of non-Bt and pyramided corn containing Trecepta® trait[§]

Planting Pattern		Trial-I			Trial-II			Trial-III			Pooled		
		No. larvae per plant[‡]	Larval growth index[‡†]	Plant injury rating[‡]	No. larvae per plant[‡]	Larval growth index[‡†]	Plant injury rating[‡]	No. larvae per plant[‡]	Larval growth index[‡†]	Plant injury rating[‡]	No. larvae per plant[‡]	Larval growth index[‡†]	Plant injury rating[‡]
Pure stand of non-Bt plants		0.20 ± 0.02 a	3.90 ± 0.23 a	5.70 ± 0.46 a	0.80 ± 0.21 a	3.41 ± 0.25 a	5.43 ± 0.51 b	0.24 ± 0.05 a	3.77 ± 0.04 a	2.49 ± 0.46 a	0.39± 0.11 a	3.72 ± 0.13 a	4.65 ± 0.53 ab
Pure stand of Bt plants		0.00 ± 0.00	N/A	1.31 ± 0.12	0.00 ± 0.00	N/A	1.00 ± 0.00	0.00 ± 0.00	N/A	1.01 ± 0.01	0.00 ± 0.00	N/A	1.11 ± 0.06
5:95RIB	Non-Bt plant	0.25 ± 0.10 a	4.25 ± 0.38 a	5.12 ± 1.45 a	0.37 ± 0.18 a	2.56 ± 0.22 a	2.96 ± 0.11 a	0.37 ± 0.02 a	4.06 ± 0.24 a	3.00 ± 0.43 a	0.33 ± 0.07 a	3.62 ± 0.30 a	3.76 ± 0.59 a
	Bt plant	0.00 ± 0.00	N/A	1.35 ± 0.13	0.00 ± 0.00	N/A	1.00 ± 0.00	0.00 ± 0.00	N/A	1.08± 0.08	0.00 ± 0.00	N/A	1.13 ± 0.07
10:90RIB	Non-Bt plant	0.09 ± 0.09 a	3.67 a	6.08 ± 0.47 a	0.18 ± 0.14 a	2.58 ± 0.58 a	3.26 ± 0.37 a	0.50 ± 0.19 a	3.74 ± 0.25 a	3.18 ± 0.50 a	0.26 ± 0.10 a	3.40 ± 0.28 a	4.18 ± 0.47 ab
	Bt plant	0.00 ± 0.00	N/A	1.60 ± 0.090	0.00 ± 0.00	N/A	1.02 ± 0.02	0.00 ± 0.00	N/A	1.01 ± 0.01	0.00 ± 0.00	N/A	1.21 ± 0.09
20:80RIB	Non-Bt plant	0.20 ± 0.07 a	4.00 a	5.61 ± 0.23 a	0.93 ± 0.29 a	3.54 ± 0.28 a	6.10 ± 0.78 b	0.39 ± 0.04 a	3.75 ± 0.05 a	3.35 ± 0.29 a	0.51 ± 0.13 a	3.76 ± 0.10 a	5.02 ± 0.45 b
	Bt plant	0.00 ± 0.00	N/A	1.60 ± 0.09	0.00 ± 0.00	N/A	1.06 ± 0.02	0.00 ± 0.00	N/A	1.00 ± 0.00	0.00 ± 0. 00	N/A	1.22 ± 0.08
30:70RIB	Non-Bt plant	0.16 ± 0.04 a	3.58 ± 0.21 a	6.29 ± 0.34 a	0.83 ± 0.20 a	3.49 ± 0.10 a	5.65 ± 0.72 b	0.33 ± 0.10 a	3.58 ± 0.12 a	2.98 ± 0.46 a	0.44 ± 0.11 a	3.55 ± 0.08 a	4.98 ± 0.51 ab
	Bt plant	0.00 ± 0.00	N/A	1.74 ± 0.09	0.00 ± 0.00	N/A	1.09 ± 0.04	0.00 ± 0.00	N/A	1.02 ± 0.02	0.00 ± 0.00	4.00 ± 0.00	1.28 ± 0.10
ANOVA		$F_{4,12} = 0.67$ $P = 0.6249$	$F_{4,8} = 1.62$ $P = 0.2600$	$F_{4,12} = 0.70$ $P = 0.6055$	$F_{4,11} = 2.72$ $P = 0.0852$	$F_{4,8} = 2.84$ $P = 0.0978$	$F_{4,11} = 7.70$ $P = 0.0033$	$F_{4,10} = 0.94$ $P = 0.4773$	$F_{4,10} = 0.89$ $P = 0.5069$	$F_{4,10} = 1.11$ $P = 0.4029$	$F_{4,41} = 1.34$ $P = 0.2717$	$F_{4,34} = 0.99$ $P = 0.4258$	$F_{4,41} = 2.94$ $P = 0.0317$

[§]Virtually no larvae and plant injury were observed on all Bt plants. Thus, data recorded from Bt plants were excluded in ANOVA.

[‡] Means followed a same letter within a column were not significantly different (Tukey HSD at α = 0.05).

[†] N/A, data are not available.

Table 2.2. Larval survival, growth, and plant injury rating (mean ± sem) of susceptible and Cry1A.105/Cry2Ab2-dual-gene resistant heterozygous genotypes of *S. frugiperda* in seed blends of non-Bt and pyramided MON 89034 corn [‡]

Planting pattern	Corn	Insect genotype	No. larvae per plant[‡]	Larval developmental index[‡†]	Plant injury Rating[‡]
Pure stand of non-Bt plants		$S_1S_1S_2S_2$	0.65 ± 0.11 a	3.78 ± 0.29 b	5.91± 0.59 a
		$R_1S_1R_2S_2$	0.41 ± 0.15 a	3.40± 0.22 b	5.35± 0.81 a
Pure stand of Bt plants		$S_1S_1S_2S_2$	0.00 ± 0.00	N/A	1.00 ± 0.00
		$R_1S_1R_2S_2$	0.00 ± 0.00	N/A	1.00 ± 0.00
10:90RIB	Non-Bt plants	$S_1S_1S_2S_2$	0.38 ± 0.08 a	3.67± 0.39 b	5.08± 0.55 a
		$R_1S_1R_2S_2$	0.39 ± 0.10 a	4.00± 0.36 b	5.27± 0.29 a
	Bt plants	$S_1S_1S_2S_2$	0.00 ± 0.00	N/A	1.00 ± 0.00
		$R_1S_1R_2S_2$	0.00 ± 0.00	N/A	1.00 ± 0.00
20:80RIB	Non-Bt plants	$S_1S_1S_2S_2$	0.45 ± 0.12 a	3.79± 0.34 b	4.37± 0.24 a
		$R_1S_1R_2S_2$	0.27 ± 0.14 a	3.50± 0.76 b	4.79 ± 0.26 a
	Bt plants	$S_1S_1S_2S_2$	0.00 ± 0.00	N/A	1.00 ± 0.00
		$R_1S_1R_2S_2$	0.00 ± 0.00	N/A	1.00 ± 0.00
30:70RIB	Non-*Bt* plants	$S_1S_1S_2S_2$	0.43 ± 0.19 a	3.06± 0.45 a	4.79± 0.37 a
		$R_1S_1R_2S_2$	0.36 ± 0.11 a	3.08± 0.20 a	4.74± 0.40 a
	Bt plants	$S_1S_1S_2S_2$	0.00 ± 0.00	N/A	1.00 ± 0.00
		$R_1S_1R_2S_2$	0.00 ± 0.00	N/A	1.00 ± 0.00
ANOVA		Insect genotype	$F_{1,20} = 3.07; P=0.0949$	$F_{1,20} = 0.09; P = 0.7689$	$F_{1,20} = 0.00; P=0.9501$
		Planting pattern	$F_{3,20} = 1.45; P=0.2570$	$F_{3,20} = 3.58 ; P = 0.0321$	$F_{3,20} = 1.51; P=0.2418$
		Interaction	$F_{3,20} = 0.88; P = 0.4682$	$F_{3,20} = 0.81; P = 0.5058$	$F_{3,20} = 0.35; P = 0.7930$

[§] Virtually no larvae and plant injury were observed on all Bt plants. Thus, data recorded from Bt plants were excluded in ANOVA.

[‡] Means followed a same letter within a column were not significantly different (Tukey HSD at α = 0.05).

[†] N/A, data are not available.

2.3.4. Larval survival of aabb and AaBb in different planting patterns of non-Bt and VT-2P corn in greenhouse tests

No live larvae were observed on VT2P plants across all planting patterns and for both aabb and AaBb insect genotypes (Table 2.2). Results from non-Bt plants showed that the effect of treatment on larval survival was not significant for insect genotype ($F_{1,20} = 3.07$, $P = 0.0949$), planting pattern ($F_{3,20} = 1.45$, $P = 0.2574$) and interaction ($F_{3,20} = 0.88$, $P = 0.4682$) (Table 2.2). An average of 0.65 ± 0.11 and 0.41 ± 0.15 larvae per plant were recovered from Pure-nonBt plants infested with aabb and AaBb, respectively. The corresponding values in the three seed blends ranged from 0.38 ± 0.08 to 0.45 ± 0.12 for aabb and from 0.27 ± 0.14 to 0.39 ± 0.10 for AaBb (Table 2.2).

2.3.5. Larval growth index of aabb and AaBb in different planting patterns of non-Bt and VT2P corn in greenhouse tests

The effect on larval growth index was significant for planting pattern ($F_{3,20} = 3.58$, $P = 0.0321$), but not significant for insect genotype ($F_{1,20} = 0.09$, $P = 0.7689$) and interaction ($F_{3,20} = 0.81$, $P = 0.5058$) (Table 2.2). Larval growth indexes on non-Bt refuge plants in 30:70RIB (3.06 ± 0.45 for aabb and 3.08 ± 0.20 for AaBb) appeared to be less ($P < 0.05$) than those recorded in Pure-nonBt (3.78 ± 0.29 for aabb and 3.40 ± 0.22 for AaBb) and the other two seed blends (3.67 ± 0.39 with aabb and 4.00 ± 0.36 with AaBb in 10:90RIB, and 3.79 ± 0.34 with aabb and 3.50 ± 0.76 with AaBb in 20:80RIB). The differences in larval growth index for any other comparisons were not significant ($P > 0.05$) (Table 2.2).

2.3.6. Plant injury rating by aabb and AaBb in different planting patterns of non-Bt and VT-2P corn in greenhouse tests

As described in the field trials, plant injury rating by *S. frugiperda* in the greenhouse tests was closely related to larval survival. No notable plant injury on Bt plants was observed for both aabb and AaBb, and across all planting patterns with a plant injury rating of 1.0 ± 0.0 (Table

2.2). The effect of treatment on plant injury rating recorded on non-Bt plants was not significant for insect genotype ($F_{1,20} = 0.00$, $P = 0.9501$), planting pattern ($P_{3,20} = 1.51$; $P = 0.2418$), and interaction ($F_{2,20} = 0.35$; $P = 0.7930$). Plant injury ratings observed in Pure-nonBt were 5.91 ± 0.59 and 5.35 ± 0.81 for plants infested with aabb and AaBb, respectively. The corresponding injury ratings in the three seed blends ranged from 4.37 ± 0.24 to 5.08 ± 0.55 for aabb and from 4.74 ± 0.40 to 5.27 ± 0.29 for AaBb.

2.4. Discussion

Previous studies have reported that Bt corn hybrids traits expressing Cry1A.105/Cry2Ab2 or Cry1A.105/Cry2Ab2/Vip3A are effective against *S. frugiperda* (Santos-Amaya et al., 2015; Horikoshi et al., 2016; Zhu et al., 2019; Niu et al., 2019). In the current study, virtually no live larvae with no or little plant injury were observed on Bt plants across all field trials and greenhouse tests. The results of this study confirmed that corn hybrids containing pyramided Bt traits were highly effective in controlling this important crop pest.

The similar larval occurrence and plant injury of aabb on non-Bt plants among the six planting patterns observed in the open field trials suggest that seed blends of non-Bt and Trecepta corn unlikely placed any notable adverse effects on the performance of the susceptible *S. frugiperda* on the non-Bt refuge plants, at least under the test conditions. The performance of r target insects in seed blends has been evaluated in a few studies. Similarly, Wangila et al. (2013) reported that larval occurrence of the sugarcane borer, *Diatraea saccharalis* (F.), on non-Bt refuge plants in seed blends was not reduced compared to a pure non-Bt corn planting. Another study by Yang et al. (2014b) reported that larval occurrence (3rd-5th instars) and ear injury of the corn earworm, *Helicoverpa zea* (Boddle), on seed blend refugia was also not reduced compared to those found on the ears of pure non-Bt plantings (Yang et al., 2014b). *D. saccharalis* is a

target pest of Bt corn in the mid-south region of the U.S. (Huang et al., 2007), while *H. zea* is a major target pest of both Bt corn and Bt cotton in the entire U.S. southern region (Yang et al., 2014b). Resistance of *H. zea* to Cry1A/Cry2A corn and cotton with field control problems has been documented in the U.S. (Dively et al., 2016; Reisig et al., 2018; Kaur et al., 2019). A limitation of the current study and the study by Yang et al. (2014b) was that both experimental designs could not test effects on the entire insect life cycle. In production corn fields, early instars of *S. frugiperda* and *H. zea* larvae usually feed on plant tissues, while mature larvae move out from plants and then drop to soil for pupation (Capinera 2017a; 2017b). Thus, both field and greenhouse trials were terminated when the majority of larvae reached the 4th instars. This was done to ensure the data collections for analysis. Additional studies need to focus the effect on late instar survival to adulthood and reproduction of the insects (Yang et al., 2014a).

The comparable performance between aabb and AaBb in the greenhouse tests across the five planting patterns of non-Bt and VT2P corn indicates that the send blends did not create more favorable environments for the dual-gene resistant-heterozygous *S. frugiperda* over its susceptible counterpart. This was the first study that evaluated performance of a dual-gene heterozygous-resistant population in seed blend plantings. A previous study reported that the AaBb genotype in sequential feedings of non-Bt and VT2P leaf tissue in a laboratory rearing outperformed aabb in two of eight feeding sequences (Zhou et al., 2018). Besides different methodologies and test environments used between the current greenhouse tests and the laboratory leaf tissue bioassays by Zhou et al. (2018), two other factors might have also contributed to the observed differences. First, plant stages used in the current studies were younger than the plants used in Zhou et al. (2018). It is well documented that expression of Bt protein expressions usually decreases as plant grows (Wang et al., 2003; Bird and Akhurst, 2004;

2005). It is also believed that plants expressing higher levels of Bt proteins could reduce survival of resistant-heterozygous genotypes in seed blends (Zhou et al., 2018). Second, the laboratory leaf tissue assays by Zhou et al. (2018) were able to measure the effects on the entire insect life cycle of the insect, while, as mentioned above, the current study assessed only early larval stages (1st to 4th instars). The different results observed among studies suggest potential risks of the seed blend refuge need to be accessed carefully before implementation of the method in the southern U.S agricultural systems (Zhou et al., 2018).

The complete control (100% mortality) of AaBb observed on VT2P plants in both pure-Bt, and seed blend plantings of the current study shows that the Cry1A.105/Cry2Ab2 dual-gene resistance in *S. frugiperda* was functionally recessive on VT2P corn plants. The recessive resistance property observed in the current study was similar to the results reported in two Brazilian Cry1A.105/Cry2Ab2-resistant populations of *S. frugiperda*, in which resistance in both populations was found to be functionally recessive on VT2P hybrids (Santos-Amaya et al., 2015; Horikoshi et al., 2016). However, two other studies by Niu et al. (2019) and Zhu et al. (2019) reported that the Cry1A.105/Cry2Ab dual-gene resistance was incompletely recessive on plants containing the same VT2P trait. The different dominance levels of resistance observed between the current study and the past two studies by Niu et al. (2019) and Zhu et al. (2019) again were most likely due to different test methods used in the studies. In Niu et al. (2019), neonates were released on corn plants at V7-V9 stages and in Zhu et al. (2019), infestations were performed at V9-V10 stages. In contrast, neonate infestations were carried out at V4-V7 plant stages in the current study. Varied dominance levels of resistance to Bt crops measured at different plant growth stages have been reported in several other target species (Huang et al., 2020). For example, resistance of the old world bollworm, *Helicoverpa armigera* (Hübner), to Cry1Ac

cotton in Australia was found to be completely recessive on 4-week old cotton (Bird and Akhurst, 2004), while it was incompletely dominant on 14-week cotton (Bird and Akhurst, 2005). The commonly observed different dominance levels for Bt crop resistance among populations of a same species indicate that there are significant interaction effects between insect genotypes and test conditions. More studies are needed to investigate such interaction effects in the future (Huang et al., 2020).

In summary, pyramided Bt corn containing VT2P or Trecepta traits were very effective against *S. frugiperda*. In this study, seed blends of non-Bt and Trecepta corn did not affect the performance of the susceptible populations on refuge plants. Seed blends of non-Bt and VT2P did not create more favorable environments for survival of the Cry1A.105/Cry2Ab2 dual-gene heterozygous genotypes over the susceptible individuals. Corn plants possessing VT2P traits likely produce a 'high-dose' toxin against *S. frugiperda* as required for the HDR strategy. While additional studies are warranted to investigate the effect of seed blends on the entire life cycle of the insect, data generated from this study should provide useful information for managing *S. frugiperda* and assessing if seed blends could be a suitable refuge planting for Bt corn IRM.

3. LARVAL MOVEMENT AND SURVIVAL OF HELICOVERPA ZEA (BODDIE) IN SEED BLENDS OF NON-BT AND BT CORN CONTAINING AGRISURE VIPTERA® TRAIT: IMPLICATIONS FOR RESISTANCE MANAGEMENT

3.1. Introduction

Insect resistance development is a major challenge for the continued success of *Bacillus thuringiensis* (Bt) crops as an effective pest management tool (Tabashnik and Carrière, 2019). To delay resistance evolution, an insect resistance management (IRM) program, known as "high dose/refuge" strategy, has been implemented in the U.S. (Ostlie et al., 1997; Matten et al., 2012). This strategy requires planting a certain proportion of non-Bt corn along with Bt corn to serve as refuge for susceptible insects. Before 2010, the IRM refuge was usually planted in strips or blocks, which is often referred to as 'structured refuge' (Ostlie et al., 1997). Planting structured refuge is somewhat complicated for growers; the operational difficulties create barriers for farmers to follow the requirements (Smith and Smith, 2014; US-EPA, 2018). For this reason, the US-EPA in 2010 approved a 5:95% (non-Bt:Bt seeds) seed blend method as another refuge option for planting pyramided Bt corn in the U.S. North Corn Belt (Matten et al., 2012). To date, the seed blend refuge has not been used in the southern region of the U.S. where Bt cotton is also planted (Matten et al., 2012).

Results of mathematical models support the use of seed blend refuge for Bt crop IRM under certain assumptions (Carroll et al., 2012; Kang et al., 2012). However, empirical data of some critical factors that can substantially affect the effectiveness of seed blend refuge are still needed to validate the credibility of these models. One of those factors is the effect of larval movement on the survival of susceptible insects in seed blend refuge. Compared to structured refuge, susceptible larvae on refuge plants in seed blends could have a higher probability to move to the

neighboring Bt plants that can cause a higher mortality to the insects (Mallent and Poter, 1992; Davis and Onstad et al., 2000; Wanglia et al., 2013; Burkness et al., 2015; Dimase et al., 2020).

The corn earworm, *Helicoverpa zea* (Boddie), is the most costly crop pest in North America (Capinera, 2017a). Recently, this pest has developed practical resistance with field control problems to both Cry1A/Cry2A corn and cotton in the U.S. (Dively et al., 2016; Reisig et al., 2018; Kaur et al., 2019). The cross-crop Bt resistance in *H. zea* has raised a significant challenge for the sustainable crop production, especially for the U.S. cotton industry (Yang et al., 2016; Reisig and Kurtz, 2018; US-EPA, 2018). The objective of this study is to evaluate larval survival and movement of *H. zea* in seed blends of non-Bt and Bt corn containing the Agrisure Viptera® 3111 trait. This Bt corn trait contains the novel Vip3A gene with a different mode of action compared to any known Cry proteins (Estruch et al., 1996; Lee et al., 2003; 2006; Kurtz, 2010). Studies have shown that Vip3A does not exhibit any cross-resistance with other Bt proteins expressed in current Cry crops (Jackson et al., 2007; Niu et al., 2013; 2014; Huang et al., 2014). In addition to the resistance of *H. zea* to Cry1A/Cry2A crops, the fall armyworm, *Spodoptera frugiperda* (F.), has also developed resistance to Cry1F corn in both South and North Americas (Storer et al., 2010; Fairs et al., 2014; Huang et al., 2014; Chandrasena et al., 2018). Because of the surge of Cry resistance, the novel Vip3A gene has recently been incorporated into many existing Cry corn and cotton varieties to manage the resistance. Unfortunately, Vip3A is the only effective Bt protein in the current Bt crop market against the Cry1/Cry2-resistant *H. zea* or *S. frugiperda* (Santos-Amaya et al., 2015; Horikoshi et al., 2016; Zhu et al., 2019; Niu et al., 2019; Kaur et al., 2019; Yang et al., 2019a). Thus, preserving Vip3A susceptibility in insects is critical for the sustainability use of Bt crop technology. Other than our current study, the only other study that evaluated larval movement of *H. zea* in seed blends was conducted in the U.S.

northern region; a study by Burkness et al (2015) investigated larval movement of *H. zea* among three plants in a row with a non-Bt corn plant in the center and two Bt plants on two sides. The current study focused on larval movement and survival of the insect in a considerably larger field plot containing 105 plants and in several seed blend rates. Results generated from this study should provide valuable information in understanding larval movement behavior of *H. zea* in seed blended corn fields, as well as, assessing potential risks and refining modeling for the use of seed blend refuge for Bt crop IRM in the U.S. southern region.

3.2. Materials and methods

3.2.1. Corn hybrids

A non-Bt corn hybrid, Agrisure®NK 1694-GT (NonBt), and a Bt corn hybrid, N60F-3111, containing Agrisure Viptera®3111 trait (VIP-3) were provided by Syngenta Crop Protection (RTP, NC, USA) for the study. VIP-3 corn contains two pyramided Bt genes, Cry1Ab and Vip3A, targeting above-ground lepidopteran pests, including *H. zea.* This corn trait also contains mCry3A, which is a Bt protein that targets below-ground rootworms, *Diabrotica spp*., and has no activity for lepidopteran species. Cry1Ab is the most commonly utilized Bt protein in corn for managing corn stalk borers and other lepidopteran moth pests, while Vip3A is a relatively new Bt mode of action. Agrisure®NK 1694-GT is genetically closely related to the Bt corn hybrid, N60F-3111.

3.2.2. Experimental designs

Three caged trials were conducted in the fields in 2018 and 2019. One trial was performed in 2018 at the Louisiana State University Agricultural Center's (LSU AgCenter) Northeast Research Station near St. Joseph, Louisiana. The other two trials were carried out in 2019 at the LSU AgCenter's Macon Ridge Research Station near Winnsboro, LA, and Dean Lee Research

Center near Alexandria, LA, respectively. Each trial consisted of eight planting patterns with five

rows separated one-meter apart and 21 plants spaced in 15.2-cm apart in a row. This equals 105

plants per plot, and each plot was separated by three meters. The eight planting patterns were 1)

pure stand of 105 non-Bt plants (Pure non-Bt); 2) pure stand of 105 Viptera 3111 plants (Pure

Bt); 3) a blend of 10% non-Bt and 90% Bt seeds with a non-Bt plant in the center (10%RIB$_{CNB}$);

4) a blend of 10% non-Bt and 90% Bt seeds with a Bt plant in the center (10%RIB$_{CB}$); 5) a blend

of 20% non-Bt and 80% Bt seeds with a non-Bt plant in the center (20% RIB$_{CNB}$); 6) a blend of

20% non-Bt and 80% Bt seeds with a Bt plant in the center (20% RIB$_{CB}$); 7) a blend of 30% non-

Bt and 70% Bt seeds with a Bt plant in the center (30% RIB$_{CNB}$); and 8) a blend of 30% non-Bt

and 70% Bt seeds with a Bt plant in the center (30% RIB$_{CB}$). Also, a non-infested control

planting of pure non-Bt seeds was included in the two trials that were conducted in 2019 to

monitor natural infestations of *H. zea*.

The pure non-Bt planting treatment was designed to simulate the structured refuge as used in

the 'high dose/refuge' IRM strategy (Ostlie et al., 1997). The three seed blend rates used are

often discussed for possible rates to be used in the southern region of the U.S. Except for the

central plants, all other seeds were randomly planted within each plot. In each seed location, two

seeds were hand-planted and the locations planted with non-Bt refuge seeds were marked with

chopsticks. After germination, plants were thinned to one plant per seed location to meet the

desired planting patterns. After three weeks, the center plant in each plot was tagged with

colored tape. Irrigation, fertilization, herbicide application, and other management practices were

performed as needed to ensure ideal plant growth. In each trial, the eight (2018) or nine (2019)

planting patterns were arranged in a randomized complete block design with four replications.

Previous observations determined a key factor that can confound field trials was natural

occurrence of *H. zea*. Thus, all field plots were caged with 8-ft high Agfabric standard insect screen nets at two weeks before the silking stage. In addition, all plots inside the cages were visually inspected for natural occurrence of *H. zea* at least three times before artificial infestations. Any larvae or adults, if found, were removed from cages to minimize any confounding effect of natural populations.

3.2.3. Field infestations

At R1 plant stage for each trial, 35 F1 neonates produced from field-collected *H. zea* were manually placed on the ear silks of the central plant in each plot except for the non-infested control plots. The use of F1 neonates produced from field-collected populations for each trial was to ensure their natural vigor of the insect. To generate F1 neonates for infestations, >300 individuals of third to fifth instars of *H. zea* were collected from non-Bt sweet corn fields near Winnsboro, Louisiana, approximately one month before each infestation. Field-collected larvae were reared individually on a meridic diet (Ward's Stonefly Heliothis diet, Rochester, NY) as described in Kaur et al. (2019). Insect development within each field collection was synchronized using varied temperatures. Pupae collected from the diet were placed in 20-L mesh cage (Seville Classics, INC., Torrance, CA) for adult emergence, mating, and egg-laying as also described in Kaur et al. (2019). F1 neonates hatched from eggs produced in the laboratory rearing were used in each trial. Number of live larvae on each ear, larval developmental stage, and kernel damage area were checked for all plants when majority of larvae on non-Bt plants reached fourth instars; larvae and ear measurements for the three trials varied from 9 to 13 d after neonate release, depending on the weather conditions. The three seed blend patterns with non-Bt plants in the centers were designed to create conditions for larval movement simulation of neonates hatched on non-Bt refuge ears. In contrast, the other three seed blends with Bt plants in

the centers were used to create conditions to simulate larval movement that originated on Bt plants. All central plants, as well as, other plants that had live larvae or showed damaged kernels were tested using an enzyme-linked immunosorbent assay technique (Quantiplate™ kits; EnviroLogix, Portland, ME) to verify expression/non-expression of Bt proteins in the plants.

3.2.4. Data analysis

Data on developmental stages of larvae recovered were converted as larval developmental index: $1 = 1^{st}$ instar, $2 = 2^{nd}$ instar, …, $6 = 6^{th}$ instar (Yang et al., 2014a). To facility data analysis, data on the number of live larvae recovered, larval developmental index, and kernel damage area observed within each plot were arranged in two ways: 1) by distance class and 2) by row. In the arrangement with distance class, data were organized as per distance class similarly as described in Wanglia et al. (2013) (Figure 3.1). Distance class 0 (DC_0) refers to the center plants that were initially infested with 35 F1 neonates/plant; Distance class 1 (DC_1) includes the eight plants that were one-plant away from the central plant; Distance class 2 (DC_2) consists of the 16 plants that were two-plants away from the center plant; Distance class 3 (DC_3) contains the ten plants that are three-plants away, and this method continues until class 10 (DC_{10}) that are ten-plants away for the center plant. Because few larvae moved and survived on ears at DC_4 to DC_{10}, data recorded from these seven distance classes were pooled in data analysis. Data were also organized based on rows: Row_0, the center row of a plot; Row_1, the two rows immediately adjacent to the center rows each in one side; and Row_2, the two rows next to the two adjacent rows (Figure 3.1).

As reported in the results, virtually no live larvae and little or no kernel damage were observed in pure Bt planting, as well as in the three seed blends with Bt plants in the centers. Thus data collected from these four planting patterns were excluded in the analysis of variance (ANOVA).
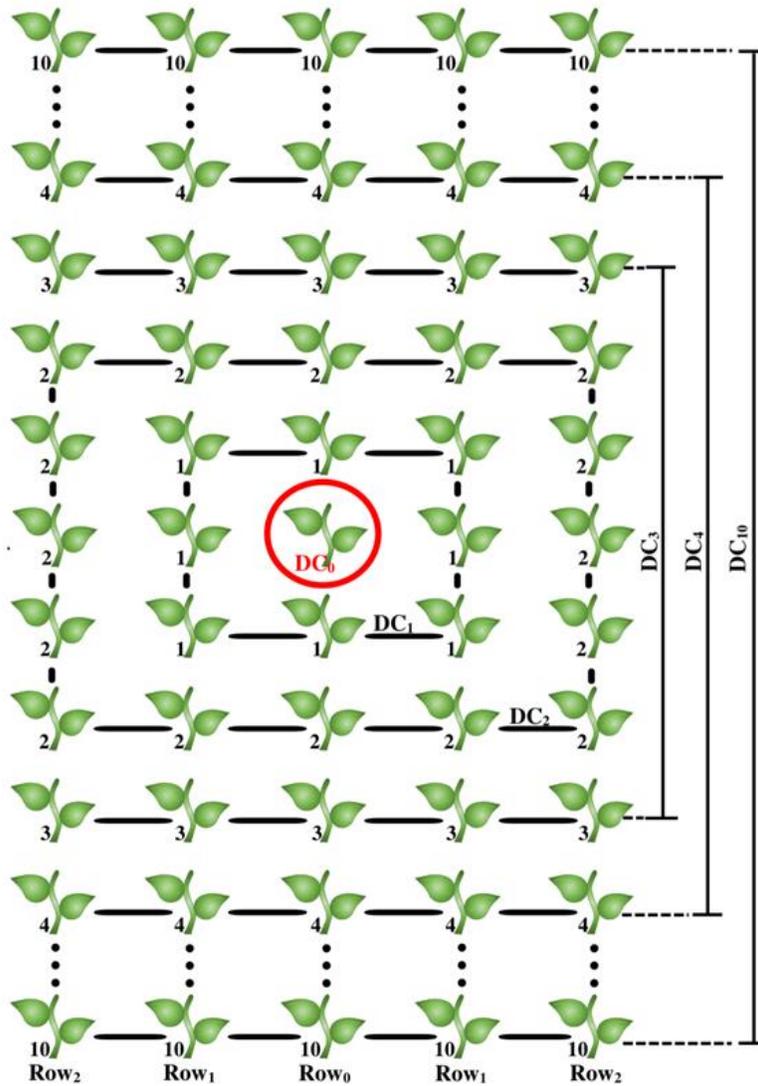
Figure 3.1. Plot diagram to illustrate data arrangement for statistical analysis. Distance class 0 (DC_0) refers to the center plants that were initially infested with 35 F1 neonates/plant; Distance class 1 (DC_1) includes the eight plants that were one-plant away from the central plant; Distance class 2 (DC_2) consists of the 16 plants that were two-plants away from the center plant; Distance class 3 (DC_3) contains the ten plants that are three-plants away, and this method continues until class 10 (DC_{10}) that are ten-plants away for the center plant. Data were also organized based on rows: Row_0, the center row of a plot; Row_1, the two rows immediately adjacent to the center rows each in one side; and Row_2, the two rows next to the two adjacent rows.

Despite the use of cages and in-field inspections, a few live larvae were still recovered from the non-infested control plots in 2019. Thus larval occurrence and kernel damage area data on non-Bt plants were corrected by subtracting the average values of natural occurrence observed in the non-infested control plots. In general, the results of the three trials were consistent. To increase the degrees of freedom in the data analysis, data collected from the three trials were pooled for each variable in ANOVA. The corrected number of live larvae per ear, larval developmental index, and kernel damage area (cm^2) per ear were transformed using log (x +1) for normal distributions and then analyzed using mixed models with planting pattern and DC/row as the two main factors, and trial as a random factor (SAS Institute, 2010). Treatment means were separated using Tukey's HSD tests at $\alpha = 0.05$ level. Untransformed data were presented in the figures.

3.3. Results

3.3.1. Performance of *H. zea* larvae on corn plants in pure stand of Viptera 3111 and three seed blends with Bt plants in the centers

Across all trials and all planting patterns, no live larvae of *H. zea* and no ear damage were found on all Viptera 3111 plants, including those central Bt plants that were initially infested with 35 neonates. In addition, across all three trials, only three larvae (one third instar, one fourth instar, and one fifth instar) with little kernel damage were observed on the non-Bt refuge plants in the three seed blends with Bt plants in the centers (data not shown).

3.3.2. Larval movement and survival of *H. zea* by distance class in pure non-Bt and three seed blends with non-Bt plants in the centers
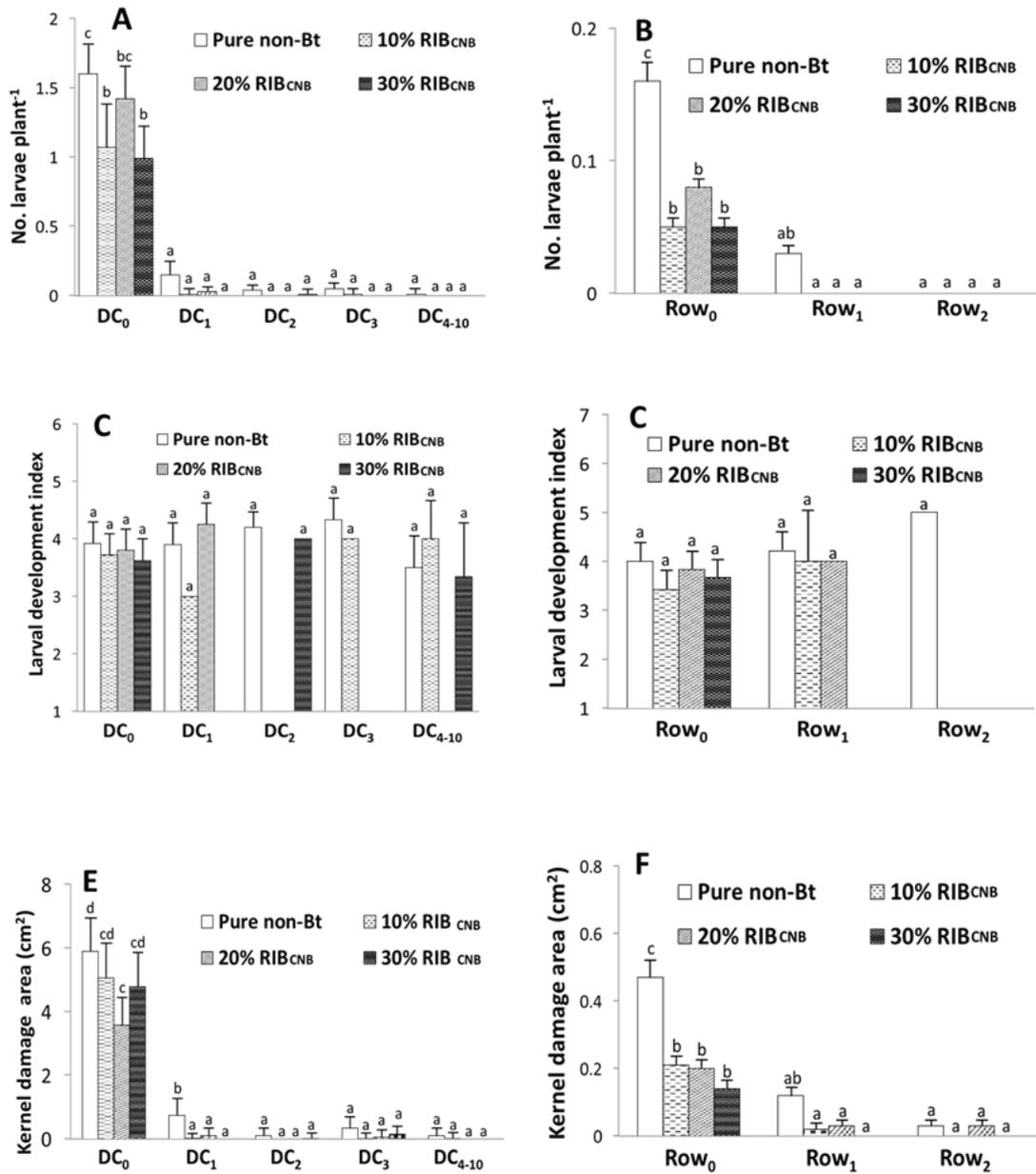
Figure 3.2. Larval occurrence and developmental index of, and ear kernel damage area (mean ± sem) by *H. zea* based on distance class (DC) or row in pure non-Bt planting and three seed blends of Cry1Ab/Vip3A corn with 10, 20, and 30% refuge and with non-Bt plants in the centers. Mean values followed by the same letter are not significantly different (Tukey's HDS test, α = 0.05). Note: virtually no live larvae were recovered from pure Bt planting and seed blends with Bt plants in the centers and thus, data recorded from those treatments were not included in the statistical analysis.

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ANOVA with data sampled from only the pure non-Bt planting and the three seed blends with non-Bt plants in the centers showed that the effect of treatment on larval occurrence was significant for planting pattern ($F_{3, 203} = 3.00$; $P = 0.0317$) and distance class ($F_{4,203} = 90.60$; $P < 0.0001$), but there was no significant interaction ($F_{12,203} = 1.25$; $P = 0.2492$). An average of 1.60 larvae/ear (or 35.2% of the total larvae recovered) of *H. zea* were found from the central plants in pure non-Bt plantings, which indicated that at least 64.8% larvae moved away from the initially infested non-Bt central plants and survived on other plants of the plots (Figure 3.2A). Among these larvae, 0.15 larvae/ear (or 40.7% of the larvae that were moved out) moved and survived on the ears of the plants at DC_1. In addition, 0.04 larvae/ear (or 21.8%) and 0.05 larvae/ear (or 17.0%) moved and survived on the ears at DC_2 and DC_3, respectively. Some larvae (0.01 larvae/ear, or 20.4%) moved and survived on the ears farther than DC_3. The differences in larval occurrence were significant ($P < 0.05$) between D_0 and other distance classes, but not significant ($P > 0.05$) among D_1 to D_{4-10} (Figure 3.2A).

Simultaneously, larval movement and survival were similar among the three seed blends with non-Bt plants in the center. A predominant number of larvae (1.15 larvae/ear or 85.7% of the total) were recovered from the central plants, while the remaining larvae (14.3%) moved away and survived on non-Bt refuge ears at DC_1 (7.9% of the total), DC_2 (3.9%), or DC_3 (2.5%). No live larvae were observed from DC_4 to DC_{10} (Figure 3.2A). Compared to the larval occurrence in pure non-Bt plantings, an overall of 27.5% fewer live larvae were recovered from the central refuge plants of the three seed blends; the differences relative to the pure non-Bt planting were significant ($P < 0.05$) for 10%RIB and 30%RIB, but not ($P > 0.05$) for 20%RIB (Figure 3.2A). Number of larvae recovered from D_1 to D_{4-10} was not significant ($P > 0.05$) among the four planting patterns (Figure 3.2A).

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3.3.3. Larval movement and survival of *H. zea* by row in pure non-Bt and three seed blends with non-Bt plants in the centers

The effect of treatment on larval occurrence was significant for planting patterns ($F_{3,118} = 6.17$; $P = 0.0006$), row ($F_{2,118} = 37.04$; $P < 0.0001$), and interaction ($F_{6,118} = 2.72$; $P = 0.0166$). In pure non-Bt planting, an average of 0.16 larvae/plant (or 72.7%) were recovered from central rows (Row_0) and 0.03 larvae/plant (or 27.3%) were found from Row_1, while no larvae were observed from Row_2 (Figure 3.2B). In seed blends, 100% live larvae were recovered from the central rows, and the number of larvae located on central rows was similar ($P > 0.05$) among the three seed blends. The average larval recovery (0.06 larvae/plant) in the three seed blends in the central rows was significantly ($P < 0.05$) less than that (0.16) recorded from the pure non-Bt planting (Figure 3.2B).

3.3.4. Larval development of *H. zea* in pure non-Bt and three seed blends with non-Bt plants in the centers

ANOVA with data organized based on distance class showed that the effect of treatment on larval development index was not significant for planting pattern ($F_{3,34} = 2.28$; $P = 0.0973$), distance class ($F_{4,34} = 2.09$; $P = 0.1038$) and interaction ($F_{7,34} = 1.12$; $P = 0.3732$). The developmental index of larvae recovered was similar regardless of planting patterns or locations, and the majority of larvae were fourth instars (Fig. 2C). Analysis with data arranged by row showed that the treatment effects were also not significant for planting pattern ($F_{3,25} = 0.57$; $P = 0.6394$), row ($F_{2,25} = 1.29$; $P = 0.2943$), and interaction ($F_{2,25} = 0.30$; $P = 0.7434$) (Figure 3.2D).

3.3.5. Kernel damage area by *H. zea* in pure non-Bt and three seed blends with non-Bt plants in the centers

Distribution of kernel damage area by *H. zea* followed similar patterns as described in the larval occurrence. Data analysis based on distance class showed that treatment effects were significant for planting pattern ($F_{3,204} = 5.51$; $P = 0.0012$) and distance class ($F_{4,204} = 258.21$; $P <$

0.0001), but not significant for interaction ($F_{12,204} = 1.36$; $P = 0.1853$). Kernel damage areas of the central plants, which was 5.89 cm^2/ear in pure non-Bt plantings, 5.06 in 10%RIB, 3.57 in 20%RIB, and 4.78 in 30%RIB, were all significantly ($P < 0.05$) greater than that observed at other distance classes across all planting patterns (Fig. 2E). The difference in kernel damage area of the central plants between pure non-Bt stands and seed blends was significant ($P < 0.05$) for 20% RIB, but not significant ($P > 0.05$) for other two RIBs; and the differences among the three seed blends were not significant ($P > 0.05$). The kernel damage (0.74 cm^2/ear) observed at DC_1 in the pure non-Bt planting was significantly greater than those (0.00-0.34 cm^2/ear) observed at a distance of $\geq DC_2$ in the pure non-Bt planting or $\geq DC_1$ in the three seed blends (Figure 3.2E). Similarly, data analysis based on row exhibited significant treatment effect for planting pattern ($F_{3,118} = 5.35$; $P = 0.0017$) and row ($F_{2,118} = 35.08$; $P < 0.0001$), but not significant for interaction ($F_{6,118} = 1.20$; $P = 0.3107$).

In general, the kernel damage area of central rows, which was 0.47 cm^2/ear in pure non-Bt and 0.14-0.21for the three RIBs, was significantly greater than those (0.00 to 0.12 cm/ear) observed in any other rows (Figure 3.2F). The kernel damage area of the central rows in pure non-Bt plantings was significantly ($P < 0.05$) greater than those observed in the three seed blends, while the differences among the seed blends were not significant ($P > 0.05$). There were also no significant ($P > 0.05$) differences in the kernel damage area between Row_1 and Row_2 across four planting patterns (Figure 3.2F).

3.4. Discussion

Two previous studies also evaluated the performance of Bt corn containing Vip3A gene against *H. zea*. Burkness et al. (2010) reported that Bt sweet corn expressing single gene Vip3A or dual genes Cry1Ab/Vip3A was highly effective against *H. zea* in field trials in Minnesota and

Maryland, U.S.A. In addition, Yang et al. (2015b) reported that no live larvae of *H. zea* with little ear damage were observed on Viptera 3111 corn in multiple trials under heavily natural pressures in Louisiana, U.S.A. Based on the results, both Burkness et al. (2010) and Yang et al. (2015b) thought that Bt corn expressing Vip3A protein likely produces a 'high dose' for *H. zea* as defined in the current adopted 'high dose/refuge' IRM strategy.

In the current study, virtually no live larvae of *H. zea* and kernel damage were observed on all Viptera 3111 plants either in pure Bt plantings or in seed blends. The result of zero live larvae recovered from all Bt plants across all seed blends also suggests that Vip3A corn could quickly kill any *H. zea* larvae that moved from non-Bt plants, even if the movement occurred at late instars. Although the 'high dose' qualification of Vip3A corn still needs to be validated with *H. zea* populations possessing major Vip3A resistance alleles as described in Huang et al. (2020), the results of the current study provided additional evidence to support the previous claim that Bt corn containing Viptera 3111 trait likely produces a 'high dose' for the insect, not only in pure stand planting but also in seed blends.

It should be emphasized that the *H. zea* populations used to produce F1 neonates for this study were collected from the area where practical resistance to Cry1A/Cry2A corn has already occurred (Kaur et al., 2019). These *H. zea* populations used in the current study are likely to be resistant to Cry1Ab protein already. Therefore, the observed efficacy of Viptera 3111 was most likely attributed to only the Vip3A protein. Additionally, the efficacy of Bt corn containing Vip3A gene against *H. zea* has also been reported in a multi-state study in the southern region of the U.S. (Reisig et al., 2015) and a recent field study by Bilbo et al., (2019). However, Yang et al. (2019b) recently reported that larval survival of *H. zea* up to third instars was commonly observed on corn plants expressing Cry1Ab/Cry1F/Vip3A in a field trial in Texas in 2018. A

laboratory strain derived from the field survivors on the Bt corn has exhibited a lower susceptibility to Vip3Aa51 in a diet over-lay bioassay relative to a susceptible strain (Yang et al., 2019b). Vip3Aa51 protein used in the bioassays by Yang et al. (2019b) could be somewhat different from the Vip3A protein expressed in the Bt corn. While additional studies are warranted to reveal the reasons that caused the less efficacy of the field control, the significant survival of *H. zea* on Vip3A-expressed corn in Texas has raised significant concerns about the durability of Vip3A crops for managing this insect.

The observed larval distributions in pure stand of non-Bt corn in the current study suggest that significant larval movements (65.4%) of *H. zea* existed in corn fields. However, the movements mainly occurred within a distance of one to three plants in the same rows, while a limited number of larvae could move and survive in the adjacent rows. Larval movement and survival rates of *H. zea* observed in the current study were somewhat greater than that (2-16%) reported in Burkness et al. (2015). Many factors, such as trial environment, insect source, plant trait, experimental design and measurement, could have contributed to the observed differences. For example, larval movement/survival in the current study were checked for all 105 plants in each plot, while the study by Burkness et al. (2015) observed the distributions in only three plants in a row for each plot. Thus, any larvae that moved farther than one-plant away or move to neighboring rows were not accounted for in Burkness et al. (2015). In particular, the two side plants in seed blend evaluated by Burkness et al. (2015) were Bt plants, which implies that any larvae moved to the side plants would likely be killed. For this reason, the authors pointed out that larval recovery from adjacent ears in the send blend could underestimate the larval movement. Moreover, Burkness et al. (2015) checked the larval movement at ten sampling dates, while the current study measured only the endpoints when the majority of the larvae reached

fourth instars. The varied results reported among studies suggest that data should be generated under different environments so that comprehensive evaluations on the effect of larval movement on seed blend refuge can be achieved.

The significant larval movement of *H. zea* should be a major factor that caused a significant reduction (27.5%) in the number of larvae hosted on refuge plants in the seed blends. There was more likely only a unidirectional movement in seed blends, in which larvae that moved out from the initially infested plants to neighboring plants would likely be killed because most neighboring plants were Bt plants, especially for highly effective Bt plants such as Vip3A corn. In contrast, larval movement could be bidirectional in pure non-Bt planting, in which larvae could move out from their initially infested plants to neighboring plants, and these larvae moved to neighboring plants also could move back to the original plants. Another critical factor for the fewer larvae hosted on refuge ears in seed blends could be due to partial expressions of Bt proteins in ear kernels of refuge plants that was commonly caused by corn cross-pollinations in seed blends (Yang et al., 2014a; Guo et al., 2019; Dively et al., 2020). Nevertheless, the observed significant reduction of *H. zea* populations hosted on seed blend refuge in this study should have important implications in improving IRM programs for the use of Bt corn traits containing Vip3A gene.

Previous studies reported that *H. zea* larvae on seed blend refuge ears developed slower than on ears of pure non-Bt corn planting (Yang et al., 2014a; 2014b; 2015b; Burkness et al., 2015). The delayed larval development in those studies was believed to be also caused by the partial expressions of Bt proteins in the refuge ears due to cross-pollination in seed blends. In the current study, the development of the larvae recovered from refuge ears in seed blends was numerically delayed relative to the larvae found from pure non-Bt plantings (Figure 3.2C). The

larval developmental delay observed in the current study was likely due to differences in the trial

conditions. As mentioned previously, to minimize confounding effects from natural infection of

H. zea, all trial plots were caged using insect nets. The cages used may have reduced air

circulation inside the plots that interfered with crosspollination. Thus, Bt protein contamination

in refuge ears would be less in caged conditions relative to open-field studies. Further, it is also

possible that reduced cross-pollination in caged environments might reduce larval mortality

caused by Bt protein contamination in refuge ears. However, previous studies reported that *H.*

zea larval occurrence up to the fourth instar was not reduced on refuge ears in seed blends (Yang

et al., 2014a; 2014b; 2015b; Burkness et al., 2015). Thus, any impact of Bt protein contamination

on larval mortality in the current study should be a minimum. All trials were terminated when

the majority of the larvae on non-Bt ears entered the fourth instar to ensure successful data

collection. Based on field observations, late instars ($\geq 3^{rd}$ instars) of *H. zea* rarely move from ears

to ears. Burkness et al. (2015) also observed that most larval movement of *H. zea* in corn fields

occurred in the early larval stages (e.g., first and second instars). Thus, the larval movement

behaviors observed in the current study should represent the general patterns of larval movement

of *H. zea* in corn fields.

Several previous studies evaluated larval movement of a few other moth pests targeted by Bt

corn. Ross and Ostlie (1990) reported that >50% neonates of the European corn borer, *Ostrinia*

nubilalis (Hübner), a primary target pest of Bt corn in North America (Ostlie et al., 1997), could

disperse in the first two days from their infested plants to other plants in corn fields, while

predominant larvae remained in the infested rows after 21 d of egg infestations. Neonates of *O.*

nubilalis could disperse more frequently from Bt corn plants than from non-Bt corn (Goldstein et

al., 2010; Razze and Mason, 2012). Davis and Onstad (2000) also stated that seed blends could

increase the dispersal of *O. nubilalis* neonate from Bt corn plants, reduce the survival of larvae that moved from Bt plants to non-Bt plants, and cause some late-instar movements from non-Bt plants to Bt plants. Wangila et al. (2013) reported that larvae of the sugarcane borer, *Diatraeainia saccharchalis* (F.), a target pest of Bt corn in South America (Grimi et al., 2018) and the mid-south region of the U.S. (Huang et al., 2007), could move at least four plants away from the infested plants, while majority of the larvae remained within a distance of three plants from the infested plants after 21 d of egg infestations. Larval dispersal of the two stalk borers is mainly through silking or walking. Bell et al. (2005) reported that *O. nubilalis* neonates usually secrete silks for dispersal. With the help of wind currents, the neonates can use the silks to hang themselves and blow them from one location to other spots (Bell et al., 2005). While additional studies are still needed to detail the dispersal behavior of *H. zea*, our preliminary laboratory and field observations suggest that *H. zea* neonates likely disperse more actively by walking than relying on the silking movement behavior as the stalk borers.

In summary, Viptera 3111 corn is highly effective and likely produces a high dose against *H. zea*. Larvae of *H. zea* in pure non-Bt corn plantings could move from the infested plants to at least four plants away, as well as to the immediately adjacent rows, but the majority of larvae distributed within a distance of three plants away in the same rows. In seed blends of Viptera 3111 with 10 to 30% refuge, predominant larvae were located on the central refuge ears that were initially infested with neonates, and a few could move to the refuge ears that were located immediately adjacent to the central plants in the same rows. Relative to pure non-Bt plantings, seed bend refuge hosted 27.5% less *H. zea* larvae than structured refuge. Distributions of ear kernel damage area in each planting pattern were closely correlated to the corresponded larval distribution, while larval development was similar across planting patterns. Data generated from

the current study should have value in understanding the larval behavior of *H. zea* in corn fields, as well as assessing the risk of resistance evolution and refining Bt corn IRM programs for managing this insect.

4. SUMMARY AND CONCLUSIONS

Fall armyworm, *Spodoptera frugiperda*, and corn earworm, *Helicoverpa zea* are two major pests that cause significant economic losses for corn and cotton growers worldwide. Transgenic corn and cotton crops expressing *Bacillus thuringiensis* (Bt) proteins have become a major tool in managing the two pests in the U.S. and several other countries. However, resistance evolution in target insect pests is a great threat to the sustainable use of the Bt crop technology. Due to the wide use of Bt crops, *S. frugiperda* has developed high resistance levels to Bt corn in several countries, and *H. zea* has also evolved high levels of resistance to Bt corn and Bt cotton in the U.S. To delay resistance development, a mandatory insect resistance management (IRM) strategy, named as 'high dose/refuge', has been implemented in the U.S. For this IRM strategy, a refuge area within the field to provide susceptible plants is vital, in which insects are able to complete a life cycle and randomly mate with rare resistant survivors from the Bt plants. Because of low compliance issues with structured refuge that has been used since 1996, a seed blend refuge has recently become an alternative method to provide susceptible insect populations in the U.S. North Corn Belt. One major concern for seed blend refuge is that larval movement of target insects could influence the effectiveness of the method. Little is known about the larval behavior of major pests such as S. *frugiperda* and H. *zea* in seed blend settings and the implications for IRM in the southern U.S. crop systems.

The main goal of the present thesis was to provide necessary information to assess whether seed blends could be an appropriate refuge strategy for Bt corn IRM in controlling S. *frugiperda* and H. *zea*, two major target-pests of pyramided Bt crops in the southern U.S. The specific objectives of this research were to: 1) determine the larval survival, development, and plant injury of susceptible S. *frugiperda* in structure refuge and seed blends; 2) evaluate the

performance of susceptible and dual-gene heterozygous-resistant populations of *S. frugiperda* in structured refuge and seed bends; and 3) understand larval movement of *H. zea* in structured and seed blend refuges, and analyze the related effect on the supply of susceptible insect populations for IRM. To accomplish the proposed objectives, in 2018 and 2019, three approaches were utilized: 1) open field trials to investigate the occurrence and plant injury of *S. frugiperda* in seed blends of non-Bt and Bt corn, 2) greenhouse tests to evaluate the performance of a Bt -susceptible and -heterozygous dual-gene resistant genotypes of *S. frugiperda* in seed blends of non-Bt and Bt corn, and 3) field caged-trials to study the larval movement and survival of *H. zea* in seed blends of non-Bt and Bt corn.

Three open field trials were conducted to determine if seed blend refuge could provide comparable levels of susceptible *S. frugiperda* (aabb) as the structured refuge. The three field trials evaluated larval survival, growth, and plant injury of aabb in six seed blends of Bt corn expressing Cry1A.105/Cry2Ab2/Vip3A (Trecepta corn) with 0-30% non-Bt refugia. Each plot consisted of four rows and 25 plants in each row (4 x 25 = 100 plants in each plot). These six planting patterns evaluated in the open field trials were: 1) pure stand of 100 non-Bt plants (Pure-nonBt), 2) pure stand of 100 Bt plants (Pure-Bt), 3) seed blend of 5 non-Bt and 95 Bt plants (5:95RIB); 4) seed blend of 10 non-Bt and 90 Bt plants (10:90RIB); 5) seed blend of 20 non-Bt and 80 Bt plants (20:80RIB); and 6) seed blend of 30 non-Bt plants and 70 Bt plants (30:70RIB). To ensure an occurrence of sufficient insect populations for data collections, two (for Trial-I), three (Trial-II), or five (Trial-III) neonates of the aabb genotype were manually released on leaves in each plant at V4-V5 plant stages. When the majority of larvae on non-Bt corn plants in each trial reached 4th instar, larval survival, growth stage, and plant injury were checked for all plants (usually 9-11 d after neonate release depending on the weather conditions). Plant injury by

S. frugiperda was measured using Davis' 1-9 rating scale, in which a rating of 1 refers to no injury, while a rating of 9 means severe foliar injury. In pure non-Bt corn plots, after 9-11 d of neonate releases on vegetative plants, an average of 0.39 larvae/plant with leaf injury ratings of 4.65 were recorded in the three field trials. In contrast, live larvae or plant injury were virtually not observed on Bt plants across all planting patterns. Performance of aabb on non-Bt plants was similar between seed blends and pure non-Bt plantings, suggesting that blend refuge might be able to provide an equivalent susceptible population as structured refugia.

To examine if seed blends created a more favorable environment for a Cry1A.105/Cry2Ab2-dual-gene heterozygous genotype (AaBb) of *S. frugiperda* over aabb. Greenhouse tests were conducted to investigate the performance of aabb and AaBb in five planting patterns of Bt seeds producing Cry1A.105/Cry2Ab2 (VT-2P corn) with 0-30% non-Bt refugia. Each planting pattern consisted of 4 rows and 14 plants in each row (56 plants per plot). The five planting patterns evaluated in the greenhouse tests were 1) pure stand of 56 non-Bt plants (Pure-nonBt), 2) pure standing of 56 Bt plants (Pure-Bt), 3) seed blend of 6 randomly planted non-Bt plants and 50 Bt plants (10:90RIB), 4) seed blend of 11 randomly planted non-Bt plants and 45 Bt plants (20:80RIB), and 5) seed blend of 17 randomly planted non-Bt plants and 39 Bt plants (30:70 RIB). In the greenhouse, when plants reach V4-V7 stages, three neonates (< 24 h old) of aabb or AaBb genotype were released on the leaves of each plant in a plot. Number of live larvae, larval growth, and plant injury ratings were recorded when the majority of larvae on non-Bt corn plants reached the 4th instar. In pure non-Bt corn plots, after 10-13 d of neonate releases on vegetative plants, an average of 0.65 larvae/plant with leaf injury ratings of 5.91 (Davis's 1-9 scale) were recorded in the greenhouse. In contrast, live larvae or plant injury were virtually not observed on Bt plants across all planting patterns. The two insect genotypes, aabb and AaBb, in seed blends

performed similarly, indicating that seed blends did not create more favorable conditions for AaBb over aabb in the test condition.

To examine the effect of larval movement on the effectiveness of seed blend refuge for Bt crop IRM, three caged-field trials were conducted to evaluate larval movement and survival of *H. zea* in eight seed blends of Cry1Ab/Vip3A corn (Viptera 3111 corn) with 0-30% non-Bt refuge. Each plot consisted of five rows and 21 plants in each row. The eight planting patterns were 1) pure stand of 105 non-Bt plants (Pure non-Bt); 2) pure stand of 105 Viptera 3111 plants (Pure Bt); 3) a blend of 10% non-Bt and 90% Bt seeds with a non-Bt plant in the center (10%RIB$_{CNB}$); 4) a blend of 10% non-Bt and 90% Bt seeds with a Bt plant in the center (10%RIB$_{CB}$); 5) a blend of 20% non-Bt and 80% Bt seeds with a non-Bt plant in the center (20% RIB$_{CNB}$); 6) a blend of 20% non-Bt and 80% Bt seeds with a Bt plant in the center (20% RIB$_{CB}$); 7) a blend of 30% non-Bt and 70% Bt seeds with a Bt plant in the center (30% RIB$_{CNB}$); and 8) a blend of 30% non-Bt and 70% Bt seeds with a Bt plant in the center (30% RIB$_{CB}$). To eliminate any confounding effects from the natural occurrence of *H. zea*, all field plots were caged with 8-ft high insect screen nets. At the R1 plant stage for each trial, 35 F1 neonates produced from field-collected *H. zea* were manually placed on ear silks of the central plant in each plot except for the non-infested control plots. Number of live larvae on each ear, larval developmental stage, and kernel damage area were checked for all plants when majority of larvae on non-Bt plants reached the fourth instar. After 9-13 d, no live larvae or kernel damages were observed on all Bt plants across all trials and planting patterns. In pure non-Bt plantings, 64.8% larvae moved away from infested ears and survived on other plants, but most larvae (86.8%) located within a distance of three plants from the central plants, and all larvae were found on the central or its adjacent rows. Larval survival and distribution were similar among seed blends with non-Bt

plants in the centers, in which predominant larvae (85.7%) located on the central plants and

100% were found on the central rows. Overall, larval recovery from central non-Bt plants in seed

blends was 27.5% less than that from pure non-Bt plantings. Kernel damage levels followed

similar patterns as larval survival, and larvae recovered developed similarly across treatments.

The results suggest that *H. zea* larvae moved significantly in corn fields, and such movement can

reduce susceptible insect populations hosted in seed blend refuge.

In conclusion, pyramided Bt corn containing VT2P or Trecepta traits were very effective

against *S. frugiperda*. Corn plants possessing VT2P traits likely produce a 'high-dose' toxin

against *S. frugiperda* as required for the 'high dose/refuge' resistance management strategy. Seed

blends of non-Bt and Trecepta corn did not affect the performance of the susceptible populations

on refuge plants. Seed blends of non-Bt and VT2P did not create more favorable environments

for survival of the Cry1A.105/Cry2Ab2 dual-gene heterozygous genotypes over the susceptible

individuals at the test conditions. Viptera 3111 corn is also highly effective and likely produces a

high dose against *H. zea.* Larvae of *H. zea* in pure non-Bt corn plantings could move from the

infested plants to at least four plants away, as well as to the immediately adjacent rows, but the

majority of larvae distributed within a distance of three plants away in the same rows. In seed

blends of Viptera 3111 with 10 to 30% refuge, larvae were predominately located on the central

refuge ears that were initially infested with neonates, and a few could move to the refuge ears

that were located immediately adjacent to the central plants in the same rows. Relative to pure

non-Bt plantings, seed bend refuge hosted 27.5% less *H. zea* larvae than structured refuge.

Distributions of ear kernel damage area in each planting pattern were closely correlated to the

corresponded larval distribution, while larval development was similar across the planting

patterns. While additional studies are warranted to investigate the effect of seed blends on the

entire life cycle of the target insects, data generated from this study should provide useful

information in assessing the risk of seed blend refuges and refining IRM programs for managing

S. frugiperda and *H. zea* using Bt crops.

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VITA

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