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## Evaluation of *Bacillus thuringiensis* corn containing pyramided traits for management of sugarcane borer, *Diatraea saccharalis* (F.)

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**EVALUATION OF *BACILLUS THURINGIENSIS* CORN CONTAINING  
PYRAMIDED TRAITS FOR MANAGEMENT OF SUGARCANE BORER,  
*DIATRAEA SACCHARALIS* (F.)**

A Thesis

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

by  
David Sindani Wangila  
B.Ed.Sc. Masinde Muliro University of Science & Technology, 2009  
May 2012

## **DEDICATION**

To Nelima,

My mothers' Mum and our mum

Your words are vivid to date.

“Cheer up son, move on. Things are vanity”

To God be the Glory.

## ACKNOWLEDGEMENTS

To begin with, I give all the glory and honor to the almighty God for enabling me all through this program; he is Ebenezer.

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## ABSTRACT

The sugarcane borer, *Diatraea saccharalis* (F.), is a major target of *Bacillus thuringiensis* (Bt) corn in the U.S. mid southern region. Corn expressing pyramided Bt proteins has recently become commercially available in the U.S. The objectives of this study were 1) to determine survival and plant injury of Cry1Ab-susceptible (Cry1Ab-SS), -resistant (Cry1Ab-RR), and – heterozygous (Cry1Ab-RS) genotypes of *D. saccharalis* on Bt corn containing single and pyramided Bt genes and 2) to assess larval movement of *D. saccharalis* in different planting patterns of non-Bt and Bt corn. One laboratory leaf tissue bioassay and two independent greenhouse trials were conducted to evaluate larval survival and plant injury on five corn hybrids. On intact plants of non-Bt corn, 43-62% larvae survived after 21 days. Larval survivorship on Cry1Ab corn was 4.7- 5.6% for Cry1Ab-SS, 29.4-32.5 % for Cry1Ab-RS, and 36.6- 45.6% for Cry1Ab-RR. In contrast, the 21-day survivorship on the two pyramided Bt corn hybrids was <5% for the three insect genotypes. Results of the leaf tissue bioassays were consistent with the greenhouse tests. Larval movement of *D. saccharalis* was evaluated in four planting patterns of non-Bt and Bt plants containing Genuity® SmartStax™ traits. The four planting patterns were: 1) pure stand of Bt corn, 2) a non-Bt corn plant surrounded by 26 Bt corn plants, 3) pure stand of non-Bt corn, and 4) a Bt corn plant surrounded by 26 non-Bt corn plants. Studies were conducted in three conditions: 1) greenhouse; 2) open field with artificial infestations of 50 eggs on the center plants; and 3) open field study with artificial infestations of 10 neonates on every plant. Larvae of *D. saccharalis* showed the ability to move from infested plants to at least four plants away and from the infested rows to adjacent rows. The results showed that the pyramided Bt corn can overcome the Cry1Ab resistance and thus should offer a means for Cry1Ab resistance management in *D. saccharalis*. Together with previous data, the

results indicate that the seed mixture strategy might be able to provide a similar refuge population of *D. saccharalis* as the structured refuge planting.

## **CHAPTER 1. INTRODUCTION**

### **1.1. Corn Production in the United States**

Field corn (*Zea mays* L.) is the most widely planted field crop in the world. It is grown commercially in more than 100 countries as a staple food or feed grain in Africa, Asia and the Americas (Maredia and Mihm, 1991). In 2010, worldwide corn production was 844 million tonnes far ahead of rice (*Oryza* spp), wheat (*Triticum* spp), and sorghum (*Sorghum* spp.) by 172, 194, and 288 million tonnes, respectively. Production in the U.S. accounted for 37.4% of the world total production in 2010 (FAO of the United Nations).

In 2011, area planted to corn in the United States was 91.921 million acres and the total harvest was 304.8 million tonnes of an estimated crop value of \$76 billion. Corn is a dominant field crop and it exceeded soybean acreage during the 2011 crop season. The 2011 corn acreage was up by 4.6% from 2010 during which 88.241 million acres were planted. Of the total area planted to corn in the United States, Bt corn accounted for over 65% in 2011 (James, 2011; NASS, 2012). Corn is a major feed grain for livestock and for ethanol production in the U.S. In the mid-southern region of the U.S, field corn also occupies a considerable acreage of the total crop land. In 2011, a total of 580,000 acres of field corn were planted in Louisiana. The total corn yield in Louisiana in 2011 was 1.93 million tonnes with a total value of \$469 million (NASS, 2012).

### **1.2. Major Corn Insect Pests**

There are various arthropod pests that damage field corn. A majority of these pests damage the above ground tissues and underground root tissues of field corn. Lepidopteran species are the major above-ground pests of corn plants, while coleopteran species are the most important pests that attack below ground plant tissues. The major lepidopteran species which damage corn in the

U.S. mid-southern region include the corn earworm (*Helicoverpa zea*), fall armyworm (*Spodoptera frugiperda* (J.E. Smith)) and a complex of corn stalk borers.

The major corn borers attributing economic loss to non-Bt corn in the mid-southern region are southwestern corn borer (*Diatraea grandiosella* Dyar) and sugarcane borer (*Diatraea saccharalis* (F)). Corn stalk borers feed on the leaf whorls leading to dead hearts during vegetative growth stages of the plant. In the second generation on corn, they bore into corn stalks and feed on softer inner tissues of the stalk. A survey reported yield loss of non-Bt corn from corn borer damage was >28% in the mid-southern region (Sankula and Blumenthal, 2004). A six-year survey from 2004-2009 in Louisiana indicated that *D. saccharalis* was the dominant corn borer in the major corn production areas of the state (Huang et al., 2011a, b).

### **1.3. *Diatraea saccharalis* (F.)**

*Diatraea saccharalis* is not native to the U.S. It was introduced during 1850's from the countries of the western hemisphere (Kelsheimer et al., 1950; Capinera, 2001). Occurrence of *D. saccharalis* has been recorded throughout the Caribbean, Central America and warmer parts of South America to Argentina (Capinera, 2001). It was introduced to Louisiana in 1855 in seed cane from South America and since then, it has spread to other states along the Gulf Coast inhabiting only warmer parts of the southern region of the U.S. (Stubbs and Morgan, 1902, Holloway et al., 1928). Larvae of *D. saccharalis* attack plants in the Poaceae family; this pest attacks sugarcane (Bessin and Reagan, 1990) and several other grasses such as corn, rice, sorghum, and Sudan grass (*Sorghum sorghum bicolor*).

Overwintering larvae of *D. saccharalis* pupate in spring and adults become active by April or May and a generation is completed in 25 days during the summer, while over 200 days are needed in winter to complete a generation (Fuchs et al., 1978). The females deposit flat and oval eggs in clusters of 20-30 eggs per egg mass. The duration of the egg stage is 4-6 days with

mean fecundity of about 700 when reared on corn leaves (Bessin and Reagan, 1990). Eggs within a cluster hatch about the same time and larvae tend to congregate in plant leaf whorls where they start feeding immediately (Capinera, 2001). Larvae of the first generation usually attack leaf whorl, mid rib, and the developing leaf tissues during vegetative plant stages, whereas during reproductive stages, larvae of the second generation usually damage stalks and ears by burrowing tunnels in them (Dekle, 1976; Flynn and Reagan, 1984; Flynn et al., 1984; Rodriguez-del-Bosque et al., 1990; Capinera, 2001)

Prior to early 1990's, *D. saccharalis* was not an economically important field corn pest in the U.S. mid-southern region (Castro et al., 2004a). It has recently become more important pest of corn in this area, especially in Louisiana and the Gulf Coast area of Texas. Beginning in the late 1990's, non-Bt field corn was heavily damaged by *D. saccharalis* in Louisiana. It has replaced *D. grandiosella* and now it is the dominant corn borer in the region (Falco et al., 2001; Reagan, 2001; Castro et al., 2004a; Huang et al., 2006).

A field survey from 2004-2009 showed that >80% of the total corn borer populations sampled across the major corn planting areas of Louisiana were *D. saccharalis* (Huang et al., 2011a, b). Severe damage by *D. saccharalis* was also reported in Texas (Porter et al., 2005; Huang et al., 2009) and some areas in western Mississippi and Arkansas (Davis et al., 1999; Castro et al., 2004a; Huang et al., 2006; Huang et al., 2011a, c).

#### **1.4. Management of Corn Stalk Borer**

Integrated pest management approaches helped manage *D. saccharalis* infestations greatly. Basic cultural practices, use of natural enemies, and host plant resistance combined with chemical pesticides in an integrated approach yielded commendable outcomes in corn borer management in the United States prior to the use of Bt corn technologies. There are a few varieties expressing host plant resistance traits which are less susceptible to *D. saccharalis* injury



(Hoisington et al., 1996; Kumar and Mihm, 1996). Studies by Maredia and Mihm 1991 on two resistant varieties 'MBRV-SWCB' and 'P23R' at the 9-11 leaf stage indicated that the two resistant varieties greatly reduced larval feeding of *D. saccharalis* due to antixenosis or antibiosis or a combination of both host plant resistance traits.

There are several biological control agents that have been historically used in controlling *D. saccharalis* in sugarcane. For example, red imported fire ants, *Solenopsis invicta* Buren, is an important natural enemy that has been documented to significantly predate on *D. saccharalis*. Some ant species, parasitoid wasps such as *Cotesia flavipes* Cameron (Hymenoptera: Braconidae), *Trichogramma* spp. (Hymenoptera: Trichogrammatidae), *Apanteles* spp. (Hymenoptera: Braconidae), *Agathis stigmaterus* Cresson (Hymenoptera: Braconidae), and some predators can reduce the number of *D. saccharalis* (Fuchs et al., 1979; Meagher et al., 1998; Capinera, 2001).

However, insecticide sprays had remained the major strategy for corn borer control before transgenic corn expressing *Bacillus thuringiensis* (Bt) proteins became commercially available (Ferré et al., 2008). Major insecticides used to control corn stalk borers included carbofuran and several pyrethroids (Baldwin et al., 2009). The timely application of the pesticides before the larvae bored and concealed themselves in the corn stalks proved to be of highly effective. Since 1999, Bt corn hybrids have been the primary method in management of *D. saccharalis* in the U.S. mid-southern region including Louisiana (Castro et al., 2004a).

### **1.5. Transgenic Bt Technology**

Rapid advancements in plant biotechnology have made it possible for scientists to transfer foreign genes to desired plant genomes. Transgenic plants (e.g. corn, cotton) containing Bt insecticidal genes are the first commercially available genetically modified Bt crops. Bt is a gram-positive, rod-shaped facultative anaerobic soil bacterium that produces specific crystalline

(Cry)  $\delta$ -endotoxins during sporulation and vegetative insecticidal proteins (Vip) during vegetative growth stages (Zakharyan et al., 1979; Gasser and Fraley, 1989; Vaeck et al., 1989; Estruch et al., 1997).

The studies by a bacteriologist Shigetane Ishiwata, , on the sotto disease that was killing vast populations of silkworms *Bombyx mori* (L.), in Japan in 1901 made him discover, isolate and name the soil bacterium (Ishiwata, 1901). A German biologist, Ernst Berliner, made a similar rediscovery while isolating the bacterium that had caused the death of a Mediterranean flour moth, *Ephestia kuehniella* (Zell), in 1911 (Berliner, 1915; Siegel, 2000; Sanahuja et al., 2011). Since the early 1920s, commercial formulations of Bt made up of spore/crystal preparations obtained from cultures in fermenters are dried and formed in granules or wettable powder that were used in sprays. Such insecticidal proteins produced by Bt have been used by farmers for insect- pest control under various trade names including Sporeine, Thuricide<sup>®</sup>, Able<sup>™</sup>, Biobit<sup>®</sup>, and Dipel<sup>®</sup> (Baum et al., 1999; Kaur et al., 2000; NPTN 2000). Pesticides with Bt formulation are considered as friendly to the environment, people, soil decomposers, pollinators, parasitoids, and wildlife. Bt toxins are highly diverse, highly effective, and relatively cheap to produce considered to insecticide sprays. These merits have made it to be the most widely used biopesticide all over the world. It is used mostly against lepidopteran, and coleopteran larvae and several dipteran pests (Baum et al., 1999; Kaur, 2000).

There are many strains of Bt and each strain produces a specific toxin that is highly specific to target pests. The primary targets of Bt are the lepidopteran species. Bt controls insects with toxins called insecticidal crystal proteins or delta endotoxins, although considered harmless to man and other non-target organisms, they are stomach poisons that must be eaten by the insect in order to be effective. After ingestion, the toxin is activated in the highly alkaline insect midgut. Complex interactions are involved in activating the Bt toxins in the insects.

It might involve Bt toxins and its metabolites/alteration of the chemistry of Bt toxins when they are expressed in a plant and when they pass through the gut of a herbivore (Hilbeck, 2002; Saxena et al., 2002; Andow and Hilbeck, 2004).

The  $\delta$ - endotoxins (Crystalline proteins) are so diverse and the first gene was completely sequenced in 1985 (Schnepf et al., 1985). Since then, many Cry genes have been sequenced and classified into various classes, subclasses and subfamilies based on the amino acid sequence similarities. The current grouping consists of 51 classes of cry proteins (Cry 1 to Cry 51), each class has several sub-classes (Cry1A, Cry1B, etc) and various sub- families (Cry1Aa, Cry1Ab, Cry1Ac, etc) (Li et al., 1991; Crickmore et al., 2012).

Genetically modified tobacco was the first plant modified to express  $\delta$ - endotoxins with Cry1Ab gene in 1987 in Belgium (Vaeck et al., 1989). Bt potatoes were first developed for the control of Colorado potato beetle (Perlak et al., 1993). In 1995, the Environmental Protection Agency (EPA) approved Bt potato as safe for human consumption. This became the first Bt crop to be approved in USA. Bt corn became commercially available in the U.S. in 1996 primarily for management of *O. nubilalis* and *D. grandiosella*. Later, more Bt corn was produced for controlling corn rootworms, *Diabrotica spp.*, *H. zea*, and *S. frugiperda*. In 2005, *D. saccharalis* was first listed as a target species of Bt corn in the U.S. (USEPA, 2005a, b). Bt corn expressing a single protein (Cry1Ab) was introduced in the U.S. southern States and commercially planted in 1999 (Buntin et al., 2000, 2004; Huang et al., 2006).

## **1.6. Bt Resistance**

The wide use of Bt corn demands an effective insecticide resistance management (IRM) plan to ensure the sustainable use of Bt corn technologies (Ostlie et al., 1997; Gould, 1998; USEPA, 1998, 2001; Baute, 2004). Resistance genes to Bt insecticides were earlier detected and reported in field populations of diamondback moth, *Plutella xylostella* (L.) (Tabashnik, 1994), and

cabbage looper, *Trichoplusia ni* (Hubner) in Canada (Janmaat et al., 2004). Major resistance genes to Bt crops have been found in laboratory selections in tobacco budworm *Heliothis virescens* (Fabricius), (Gould et al., 1995, 1997), pink bollworm, *Pectinophora gossypiella* (Saunders) (Tabashnik et al., 2000), poplar leaf beetle, *Chrysomela populi* (L.) (Génissel et al., 2003), *D. saccharalis* to Cry1Ab corn (Huang et al., 2007a, b, 2008, 2009), *O. nubilalis* to Cry1F corn (Pereira et al., 2008), *H. zea* to Cry1Ac cotton in the U.S (Tabashnik et al., 2008; Moar et al., 2008) and *Helicoverpa armigera* (Hübner) to Cry1Ac cotton in Australia (Akhurst et al., 2003; Downes et al., 2007; Mahon et al., 2007) and China (Li et al., 2004; Xu et al., 2009).

Field resistance in target insect species to Bt crops that leads to control failure or reduced control efficacy (Tabashnik et al., 2003; Huang et al., 2011c) has been documented in four cases. These four cases include resistance of *S. frugiperda* to Cry1F corn in Puerto Rico in 2006 (Storer et al., 2010), resistance of African stem borer, *Busseola fusca* (Fuller), to Cry1Ab corn in South Africa in 2007 (Van Rensburg, 2007), resistance of *P. gossypiella* to Cry1Ac cotton in western India (Dhurua and Gujar, 2011) and recently resistance of western corn rootworm, *Diabrotica virgifera virgifera* LeConte to Cry3Bb1 corn in Iowa, USA (Gassmann et al., 2011). Several reasons might have led to the evolution of resistance in the four cases named above. First, high selection pressure due to the wide scale and increased rates of adopting Bt crops, secondly is the planting of some hybrids that did not produce a high dose of Bt proteins against the target insect pests, and failure to comply to planting refuge areas (Huang et al., 2011b).

### **1.7. Bt Resistance Management**

The “*high dose/ structured refuge*” *IRM strategy*: Since commercialization of Bt corn, USA and Canada have been following a “high/dose structured refuge” IRM strategy for planting Bt corn. One of the requirements of this strategy is that Bt corn plants produce a high level of Bt proteins that kills Bt resistant heterozygotes of the target pests (USEPA, 2001).

Based on the definition of U.S. EPA, a Bt corn hybrid can be considered as “high dose” if it kills  $\geq 95\%$  resistant heterozygotes of the target pests (FIFRA Scientific Advisory Panel, 1998, USEPA, 2001). Furthermore, the IRM strategy involves planting a portion of corn in an area (e.g. a farm) with non-Bt corn as refuge for susceptible insects (USEPA, 2001).

The strategy takes advantage of insect movement and moth dispersal between Bt to non-Bt refuge plants. The emerging resistant insects survived from Bt corn plants mate with the susceptible insects in the refuge plants such that most offspring carrying resistance alleles will be heterozygous. The heterozygous individuals should be killed by high dose Bt corn. Therefore, resistance allele frequency in the field populations can be maintained at a low level for a long period of time (Ostlie et al., 1997). In the U.S. outside the cotton-producing regions, the “high dose/structured refuge” IRM strategy requires Bt corn growers to plant at least 20% (for single-gene expressing Bt corn) or 5% (for pyramided Bt corn) non-Bt refuge corn. In the corn-cotton overlapping regions, a minimum of 50% (for single-gene Bt corn) or 20% (for pyramided Bt corn) non-Bt refuge corn is required. Refuge plants are to be within 800 m from the Bt corn field (USEPA, 2001, Monsanto, 2010a).

Evaluations of various Bt corn hybrids in Louisiana have indicated differential performance of Bt corn against *D. saccharalis* (Castro et al., 2004b; McAllister et al., 2004; Wu et al., 2007; Ghimire et al., 2011). For instance, studies have shown that some of the single-gene Cry1Ab corn hybrids do not express a high dose against *D. saccharalis* as required by the “high dose/refuge” IRM strategy (Wu et al., 2007; Ghimire et al., 2011). Greenhouse tests have shown a significant larval survival rate of the heterozygotes of Cry1Ab resistant *D. saccharalis* on single-gene (Cry1Ab or Cry1F) Bt corn hybrids, especially during the reproductive plant stages (Wu et al., 2007; Ghimire et al., 2011; Huang et al., 2011a; Huang et al., 2012). However, recent studies showed that the Cry1Ab-resistant strain of *D. saccharalis* could not survive on transgenic

corn (event MON89034) containing pyramided Bt genes of Cry1A.105 and Cry2Ab2 (Ghimire et al., 2011).

A producer's compliance for the structured refuge requirements has been a problem. During the earlier years of Bt corn commercialization, a relatively high rate of compliance (e.g. 86-92%) to the refuge requirement was reported for U.S. growers (AGBSTC, 2005, USEPA, 2010). Unfortunately, compliance rates dropped to 74-80% in 2007 and 2008. Similar declining trend in the structured refuge planting was also reported in Canada. The refuge compliance slipped from 85% in 2003 to 61% in 2009 (Dunlop, 2009).

During the 2010-2011 crop seasons, transgenic corn technologies (e.g. Genuity<sup>®</sup> SmartStax<sup>™</sup>, Agrisure<sup>®</sup> Viptera<sup>™</sup> 3111) expressing more than one dissimilar Bt proteins that target lepidopteran pests were first commercially planted in the United States. The use of pyramided Bt corn hybrids is expected to delay resistance development in target insect populations. Because of the compliance issues in the use of “structured refuge” for resistance management, the U.S. EPA approved a seed mixture refuge strategy (also called “refuge-in-the-bag” or RIB) for planting pyramided Bt corn hybrids in the north U.S. Corn Belt where no cotton is planted (USEPA, 2010). For the RIB strategy, a portion of non-Bt corn seeds is mixed with Bt corn seeds in each bag by seed industries prior to being sold to farmers. Farmers just buy the premixed seeds and plant in their fields (Monsanto, 2011). Therefore, compliance by farmers to the refuge requirement will be no longer an issue. In structured refuge, the dispersal of adult moths is so essential (Gould, 1994; Ostlie., 1997; Shelton et al., 2000; Qureshi et al., 2006) but for the sake of “RIB” strategy it is the larvae that matters. Since the refuge is imbedded within the same field in the “RIB” strategy and given that adults lack preference for oviposition sites (Hellmich et al., 1999), it is the larval dispersal behavior that matters. Therefore, the major concern related to the use of the “RIB” strategy is larval movement among Bt and non-Bt plants

which may create a more favorable environment for resistance development in target pest populations. For example, movement of susceptible larvae from non-Bt refuge plants to Bt plants in RIB strategy could cause a greater mortality to susceptible insects than in structured refuge planting and result in a lower refuge population (Davis and Onstad, 2000; Shelton et al., 2000). On the other hand, heterozygous - resistant individuals or those insects containing minor resistance alleles could feed on non-Bt plants first and later move to the Bt plants and survive because late-instars of corn borers are much less susceptible to Bt toxins (Huang et al., 1999; Walker et al., 2000; Huang et al., 2006). Therefore, the differential susceptibility among instars and larval movement among Bt and non-Bt plants could create a sub-lethal dose exposure of target pests and promote build-up of resistance in target pest populations. Furthermore, pollen contaminations from non-Bt to Bt plants may also create sub-lethal exposures in fields having non-Bt plants planted in close proximity with Bt plants leading to cross pollination (Burkness, 2011). For these reasons, the seed mixture refuge strategy was not considered an appropriate IRM strategy for single-gene Bt corn (USEPA, 2001), although it was also discussed as an potential strategy prior to the commercial use of Bt corn (USEPA, 2001). A few models have shown that “RIB” could be an effective IRM strategy for planting pyramided Bt corn (GH in press). However, field data to support the “RIB” strategy for planting pyramided Bt corn are still very limited (Manyangarirwa et al., 2006; Alyokhin, 2011; Onstad et al., 2011).

In situations where insect movement is independent of presence of toxin inside plants, seed mixtures of Bt and non-Bt corn would appropriately be used to delay resistance development for Bt crops (Mallet and Porter, 1992). On the other hand, larval dispersal could be density dependent leading to migration from non- Bt plants to Bt plants where population density is lower (Kumar, 2004). When Bt-susceptible insects move from non-Bt to Bt plants, they may die and the proportion of insects in a pure stand of non- Bt plants might always remain higher

than in mixed seed plots. In addition, Bt- resistant heterozygotes might survive the Bt toxins after sub-lethal exposure on Bt plants followed by movement to non- Bt plants (Davis and Onstad, 2000; Shelton et al., 2000). Resistance to Bt toxins could be posed with great risks because of interplant movement of insects if it becomes a common event (Davis and Onstad 2000; Ferré et al., 2008; Onstad et al., 2011). Up to date, data to support the “RIB” strategy for the pyramided Bt corn are still very limited (Manyangarirwa et al., 2006; Alyokhin, 2011). There are several studies that have investigated the movement pattern of *O. nubilalis* (Ross and Ostlie, 1990; Davis and Coleman, 1997; Gore et al., 2002; Moreau and Bauce, 2003; Goldstein et al., 2010). In contrast, most studies on *D. saccharalis* has only centered on susceptibility to Bt toxins. No studies have been conducted to evaluate larval movement of *D. saccharalis* on Bt corn and non-Bt plants.

*Gene-pyramiding:* Pyramided Bt corn hybrids are products containing two or more Bt toxins that are effective against the same pest (USEPA, 2001). The assumption here is that the pyramided toxins (Cry proteins) have distinct and non-cross reacting modes of action against target insect pests. Therefore, the chances of a resistant insect that has multiple mutations effective against different toxins are decreased greatly (Zhao et al., 2003; Bravo et al., 2007). For instance, when the toxins bind to different receptor molecules produced in the same plant then an insect must undergo multiple mutations at one time to overcome these toxins. On the other hand, “stacked Bt hybrids” refers to products that have combined toxins targeting different pests. The majority of the first generation Bt corn expresses only a single Bt protein for a target insect pest. For example, the two most common Bt corn traits YieldGard® and Herculex® I contain only Cry1Ab and Cry1F, respectively. Both Bt toxins target above-ground lepidopteran pests, primarily corn stalk borers. Modeling has shown that insect pests could develop resistance more



rapidly to single protein Bt crops than to multiple Bt proteins (Roush, 1998; Onstad et al., 2002; Zhao et al., 2003; Monsanto, 2010b; Onstad et al., 2011; Carroll et al., 2012).

The first two commercialized pyramided Bt corn technologies in the U.S. for managing lepidopteran pests are Genuity<sup>®</sup> VT Triple Pro<sup>™</sup> and Genuity<sup>®</sup> SmartStax<sup>™</sup>. Both were first commercially planted during the 2010 crop season (Monsanto, 2010b; USEPA, 2010). Genuity<sup>®</sup> VT Triple Pro<sup>™</sup> expresses three Bt proteins, Cry1A.105, Cry2Ab2, and Cry3Bb1, among which Cry105 and Cry2Ab2 target above-ground lepidopteran pests, while Cry3Bb1 targets underground rootworms. The Genuity<sup>®</sup> SmartStax<sup>™</sup> technology contains six Bt proteins and traits for herbicide tolerance (Liberty and glufosinate). The six Bt proteins in Genuity<sup>®</sup> SmartStax<sup>™</sup> are Cry1A.105, Cry2Ab2, and Cry1F for controlling above-ground lepidopteran pests (e.g. corn borers, earworms, armyworms) and Cry3Bb1, Cry34Ab1, and Cry35Ab1 for managing rootworms (Gatehouse, 2008). The use of pyramided Bt corn technologies is expected to slow resistance development in field populations considerably (Monsanto, 2010b). Scientific data that can support the use of seed mixture as a refuge strategy for management of *D. saccharalis* with pyramided corn technology is limited.

## **1.8. Objectives**

1. To evaluate larval survival and plant injury of Cry1Ab-susceptible, -resistant, and -heterozygous genotypes of *D. saccharalis* on transgenic corn containing single or pyramided Bt genes; and
2. To investigate larval occurrence and movement of *D. saccharalis* in different planting patterns of non-Bt and Genuity<sup>®</sup> SmartStax<sup>™</sup> corn.

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Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance  
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## **CHAPTER 2. LARVAL SURVIVAL AND PLANT INJURY OF CRY1AB-SUSCEPTIBLE, -RESISTANT, AND -HETEROZYGOUS GENOTYPES OF THE SUGARCANE BORER ON TRANSGENIC CORN CONTAINING SINGLE OR PYRAMIDED BT GENES**

### **2.1. Introduction**

The sugarcane borer, *Diatraea saccharalis* (F), is a major target species of transgenic corn expressing *Bacillus thuringiensis* (Bt) proteins in South America and the mid-southern region of U. S. (Dow AgroSciences, 2009). The first generation larvae of *D. saccharalis* attack leaf whorl, mid rib and the developing leaf tissues during vegetative plant stages, whereas during reproductive stages, second generation larvae damage stalks and ears by burrowing tunnels in them (Dekle, 1976; Flynn and Reagan, 1984; Flynn et al., 1984; Rodriguez-del-Bosque et al., 1990; Capinera, 2001). Initially a major pest of sugarcane, *D. saccharalis* has expanded its host and geographic range to other grasses in the family Poaceae. In many areas of the U.S. gulf coast region, it has recently replaced the southwestern corn borer, *Diatraea grandiosella* (Dyar), as the dominant corn borer species (Falco et al., 2001; Reagan, 2001; Castro et al., 2004a; Porter et al., 2005; Huang et al., 2012). A field survey from 2004-2008, indicated that *D. saccharalis* represented >80% of the total corn borer populations in the major corn planting areas in Louisiana (Huang and Leonard, 2008; Huang et al., 2009).

Since 1999, transgenic corn expressing Bt proteins has been successfully used for management of a complex of corn stalk borers including *D. saccharalis* in the U.S. mid-southern region (Davis et al., 1999). Resistance development in target insect species is a major concern for the sustainable use of the transgenic Bt crops (Ostlie et al., 1997; Gould, 1998; USEPA, 2001; Baute, 2004; Castro, et al., 2004b). Field resistance in target insect species to Bt crops that results in control failure or reduced control efficacy has been documented in four cases.

These four cases include resistance of fall armyworm, *Spodoptera frugiperda* (J.E smith) to Cry1F corn in Puerto Rico in 2006 (Storer et al., 2010), resistance of African stem borer, *Busseola fusca* (Fuller), to Cry1Ab corn in South Africa in 2007 (Van Rensburg, 2007), resistance of pink bollworm, *Pectinophora gossypiella* (Saunders) to Cry1Ac cotton in western India (Dhurua and Gujar, 2011), and recently resistance of western corn rootworm, *Diabrotica virgifera virgifera* LeConte, to Cry3Bb1 corn in Iowa, U.S. (Gassmann et al., 2011).

A previous study reported that some of the single gene Bt corn (e.g. Cry1Ab corn) that was commonly planted in the mid-southern region did not express a high dose against *D. saccharalis* as required for in the “high dose/structured refuge” IRM strategy. Recently, Ghimire et al. (2011) evaluated six other Bt corn hybrids including four Cry1Ab (YieldGard<sup>®</sup>) and two Cry1F (Herculex<sup>®</sup>) corn hybrids against Cry1Ab-susceptible and -resistant strains of *D. saccharalis*. The results showed that all six Cry1Ab corn hybrids did not express a high dose for *D. saccharalis*. However, the Cry1Ab-resistant strain of *D. saccharalis* could not survive on two experimental corn lines (event MON89034) containing pyramided Bt genes of Cry1A.105 and Cry2Ab2 (USEPA, 2001; Ghimire et al., 2011). Gene-pyramiding is a novel strategy that has been currently employed to develop transgenic plants that express multiple Bt proteins targeting a same group of insect pests. The first two commercialized pyramided Bt corn technologies in the U.S. for managing lepidopteran pests are Genuity<sup>®</sup> VT Triple Pro<sup>™</sup> and Genuity<sup>®</sup> SmartStax<sup>™</sup>. Both were first commercially planted during the 2010 crop season (Monsanto, 2010b; USEPA, 2010). The objective of this study was to evaluate the larval survival and plant injury of Cry1Ab-susceptible, -resistant, and -heterozygous genotypes of *D. saccharalis* on corn hybrids containing single and pyramided Bt genes and thus to determine if the novel pyramided Bt corn could overcome the Cry1Ab-resistance in *D. saccharalis*.

## **2.2. Materials and Methods**

### **2.2.1. Insect Sources**

Three genotypes of *D. saccharalis* were tested in this study, which included a Cry1Ab-susceptible (Cry1Ab-SS), a Cry1Ab-resistant (Cry1Ab-RR), and F1 heterozygous (Cry1Ab-RS) genotypes. The Cry1Ab-SS strain was established from larvae collected from non-Bt plants near Winnsboro in Franklin Parish in northeast Louisiana (32° 8' 6''N, 91° 41' 18'') in 2009. The Cry1Ab-SS strain has been documented to be susceptible to purified Cry1Aa, Cry1Ab, Cry1Ac, Cry1A.105, and Cry2Ab2 proteins (Huang et al., 2012) as well as to Bt corn leaf tissue expressing Cry1Ab, Cry1A.105, and Cry2Ab2 (Huang et al., 2011a). The Cry1Ab-resistant strain was obtained from a single two-parent family developed through a F<sub>2</sub> screen in 2004 (Huang et al., 2007a). The resistant strain has demonstrated to be able to survive and complete entire larval development (from neonate to pupa) on commercial Cry1Ab corn plants in the greenhouse (Huang et al., 2007c). Before the Cry1Ab-RR strain was used in this study, it had been backcrossed with the Cry1Ab-SS strain two times and reselected for Cry1Ab resistance on Cry1Ab corn leaf tissue in the F<sub>2</sub> generations. The Cry1Ab-RS was developed from a cross between Cry1Ab-SS and the backcrossed- reselected Cry1Ab-RR.

### **2.2.2. Corn Hybrids**

Three Bt and two non-Bt commercial corn hybrids produced by Monsanto Company (St. Louis, MO) were evaluated in two independent trials during 2010 and 2011 (Table 2.1). The three Bt corn hybrids were DKC 67-23 RR2 containing YieldGard<sup>®</sup> trait, DKC 67-88 expressing Genuity<sup>®</sup> VT Triple Pro<sup>™</sup> traits (Monsanto, 2007) and DKC 61-21 SS/RR/L<sub>1</sub> possessing Genuity<sup>®</sup> SmartStax<sup>™</sup> traits. YieldGard<sup>®</sup> contains a single Bt gene, Cry1Ab. The pyramided Bt corn hybrids were recently approved for planting; before then, YieldGard<sup>®</sup> corn was the most commonly planted Bt corn technology for corn stalk borer control in the U.S. including the

mid-southern region. Genuity® VT Triple PRO™ is a pyramided Bt corn that expresses three Bt genes including Cry1A.105 and Cry2Ab2 for controlling above-ground lepidopteran pests and Cry3Bb1 for managing underground rootworms, *Diabrotica spp.* Genuity® SmartStax™ corn contains all Bt genes expressed in Genuity® VT Triple Pro™ in addition to Cry1F targeting lepidopteran species and Cry34Ab1/Cry35Ab1 targeting rootworms (Gatehouse, 2008; Monsanto, 2010 a; Monsanto, 2011). The two non-Bt corn hybrids were DKC 61-22 and DKC 67-86. The hybrid, DKC 61-22 was genetically closely related to the Bt corn hybrid, DKC 61-21, while DKC 67-86 was closely related to the Bt corn hybrids DKC 67-23 and DKC 67-88 (Table 2.1). Seed planting and plant management procedures in the greenhouse were similar to those described in Wu et al., 2007. Two seeds of a hybrid were planted in each 18.9-liter plastic pot which contained ≈ 5 kg of standard potting soil mixture (Perfect Mix™, Expert Gardener products, St. Louis, MO) in a greenhouse located in Baton Rouge, LA. The pots were kept on tables in the greenhouse allowing a proper distance from pot to pot. A mixture of southern turf builder, lawn fertilizer containing 2% iron and 32N-0P-10K (Scotts company, OH) and the Lawn and Garden plant food containing 13N-13P- 13K (Meherrin fertilizer, Inc, NC) were applied at V2 and V8 plant growth stages (Ritchie et al., 1993). Topping-up and irrigation among other management practices were availed when needed to ensure optimum growth. Expression of Bt proteins in the corn hybrids was confirmed using the ELISA-based technique (EnviroLogix, Quantiplate™ kits, Portland, ME).

### **2.2.3. Leaf Tissue Bioassay**

In 2011, fully expanded leaves at V6-V8 stages of corn plants (Ritchie et al., 1993) were removed from greenhouse grown plants and used in the laboratory bioassays. Leaf tissue bioassays were carried out in the laboratory following methods similar to those described in Huang et al. (2006) and Ghimire et al. (2011).



**Table 2.1.** Traits, Bt genes and major target species of two non-Bt and three Bt corn hybrids evaluated in the greenhouse and leaf tissue bioassay studies

Corn Hybrid	Trait and Abbreviation	Bt Event(s)	Bt genes	Major target pests
DKC67-86	Non-Bt (NonBtY)	NBt	-	-
DKC61-22	Non-Bt (NonBtS)	NBt	-	-
DKC67-23	YieldGard® (YGCB)	MON 810	Cry1Ab	Corn borers
DKC67-88	Genuity® VT Triple Pro™ (VT3P)	MON89034, MON88017	Cry1A.105,Cry2Ab2,Cry3Bb1	Stalk borers, corn earworm, armyworms, and rootworms
DKC61-21	Genuity® SmartStax™ (SMT)	MON89034, MON88017, TC1507, DAS-59122-7	Cry1A.105,Cry2Ab2,Cry1F, Cry3Bb1, Cry34/35Ab1	Stalk borers, corn earworm, armyworms, and rootworms

In the bioassay, six pieces of leaf tissue ( $\approx 20 \text{ cm}^2$ ) of a corn hybrid were placed in each well of the 8-well trays (Bio-Smart-8, C-D International, Pitman, NJ). Twenty-five neonates of each of the three genotypes of *D. saccharalis* were then placed in each well of separate trays. The wells containing leaf tissues and larvae were covered using the pull n' peel tabs (Bio- CV-1, C-D International, Pitman, NJ). Bioassay trays were placed in a growth chamber maintained at  $28^\circ\text{C}$ , a 14:10 L: D cycle and humidity of 40- 45%. Leaf tissues were replaced with fresh ones after 3 days. Larval survival was recorded on the 6<sup>th</sup> day after release of neonates. There were four replications ( $n = 100$ ) for each combination of corn hybrids and insect genotypes.

#### **2.2.4. Intact Plant Tests in the Greenhouse**

Two independent trials were conducted in the greenhouse to evaluate the larval survival and plant injury of three genotypes of *D. saccharalis* on intact plants. In each trial, 20 (trial one in 2010) or 10 (trial two in 2011) neonates (<24 h old) of each of the three insect genotypes were manually placed into the collar of the leaf directly above or below the uppermost ear at the reproductive plant stages (R1-R2) (Ritchie et al., 1993) using a soft brush (size 10/0; Daler-Rowney Ltd., Bracknell, England). Each treatment combination of corn hybrids and insect genotypes was replicated four times in a randomized complete block design in each trial. There were four plants (two pots, each with 2 plants) for each replication. Number of insect survivors in each plant, number of entry/exit holes, and tunnel length inside each stalk were recorded after 21 days of larval release.

#### **2.2.5. Data Analysis**

Larval survival recorded on leaf tissues in the laboratory bioassay and on intact plants in the greenhouse tests were transformed using arcsine ( $x^{0.5}$ ) to normalize the treatment variances. Whereas, the number of entry/exit holes and tunnel length inside stalks were transformed to

log(x + 1) scale (Zar, 1984). In each of the above cases, the transformed data were then analyzed using two-way ANOVA (SAS Institute, 2010) with insect genotype and corn hybrid as the two main factors. Treatment differences were determined using LSMEANS tests at  $\alpha = 0.05$  level of significance. The untransformed data and standard errors of the means (SEM) are presented in the figures.

Additionally, the dominance level ( $D_{ML}$ ) for Cry1Ab resistance in *D. saccharalis* was calculated using the following formula (Roush and McKenzie, 1987; Liu and Tabashnik, 1997; Bourguet et al., 2000):

$$\text{Dominance } (D_{ML}) = \frac{\text{RS Survival} - \text{SS Survival}}{\text{RR Survival} - \text{SS Survival}}$$

Where, **RR**, **RS** and **SS** refer to the three insect genotypes, resistant, heterozygous and susceptible, respectively. The level of effective dominance ( $D_{ML}$ ) ranges between 0 (completely recessive resistance) and 1 (completely dominant).

## 2.3. Results

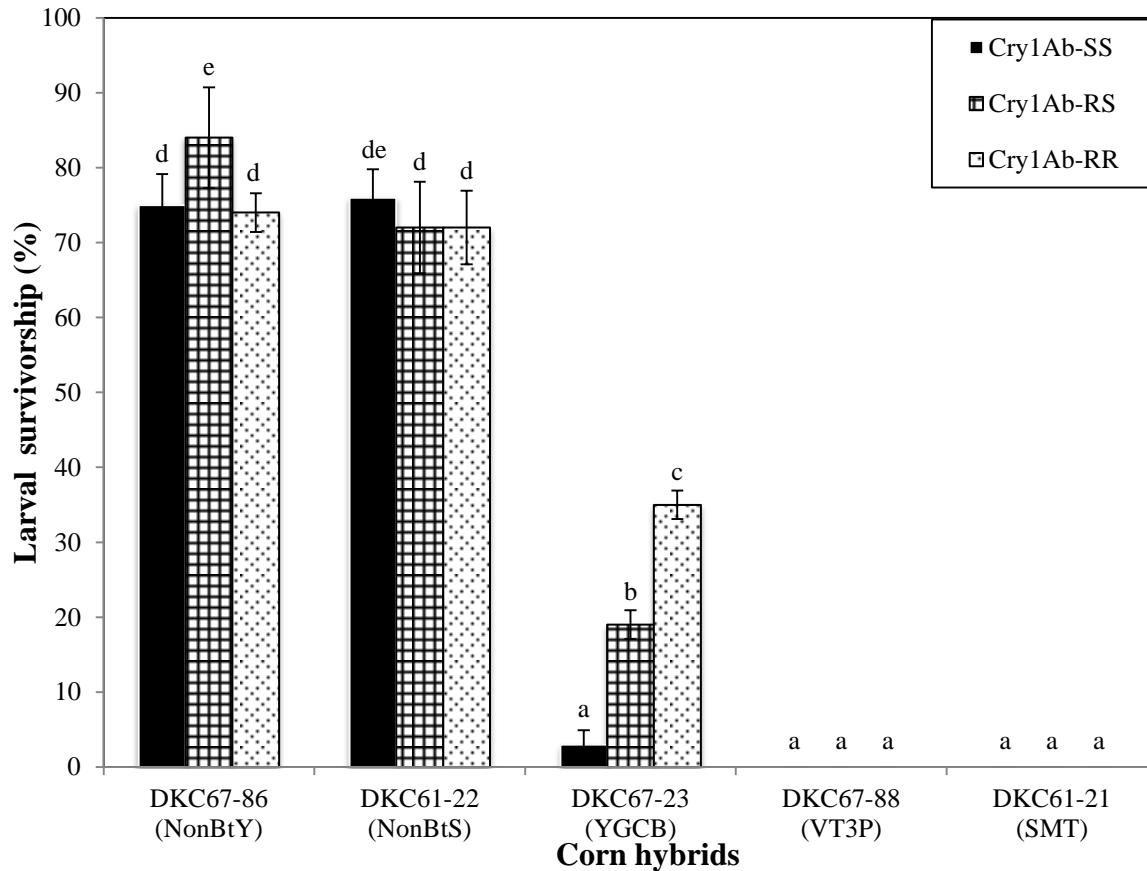
### 2.3.1. Larval Survival of Cry1Ab-SS, Cry1Ab-RS, and Cry1Ab-RR Genotypes of *D. saccharalis* on Leaf Tissues of Two Non- Bt and Three Bt Corn Hybrids

The effects of corn hybrid, insect genotype, and their interaction on 6-day larval survivorship of *D. saccharalis* were significant for all factors ( $F = 454.8$ ;  $df = 4, 42$ ;  $P < 0.0001$  for corn hybrid;  $F = 6.74$ ;  $df = 2, 42$ ;  $P = 0.0029$  for insect genotype; and  $F = 8.07$ ;  $df = 8, 42$ ;  $P < 0.0001$  for interaction). Larval survival of *D. saccharalis* on non-Bt leaf tissue was not significantly different ( $P > 0.05$ ) between the two corn hybrids and across the three insect genotypes with an average survivorship of 75.5% after 6 days (Fig. 2.1). Larval survivorship of the three insect genotypes on Bt corn leaf tissue was significantly ( $P < 0.05$ ) less than that on the

non-Bt corn leaf tissue. Only a very low survivorship (3%) of Cry1Ab-SS larvae was observed after 6 days on leaf tissue of YieldGard® plants. However, larvae of Cry1Ab-RR on YieldGard® corn leaf tissue demonstrated a 35% survivorship, which was significantly greater than that of Cry1Ab-SS. In addition, an average of 19% larvae of Cry1Ab-RS genotype also survived after 6 days on YieldGard® corn leaf tissue, which was significantly ( $P < 0.05$ ) less than that of Cry1Ab-RR but significantly ( $P < 0.05$ ) greater than that of Cry1Ab-SS. Both pyramided Bt corn hybrids were excellent against *D. saccharalis*. All larvae were killed after 6 days on leaf tissue removed from the two pyramided Bt corn hybrids regardless of the insect genotypes (Fig. 2.1).

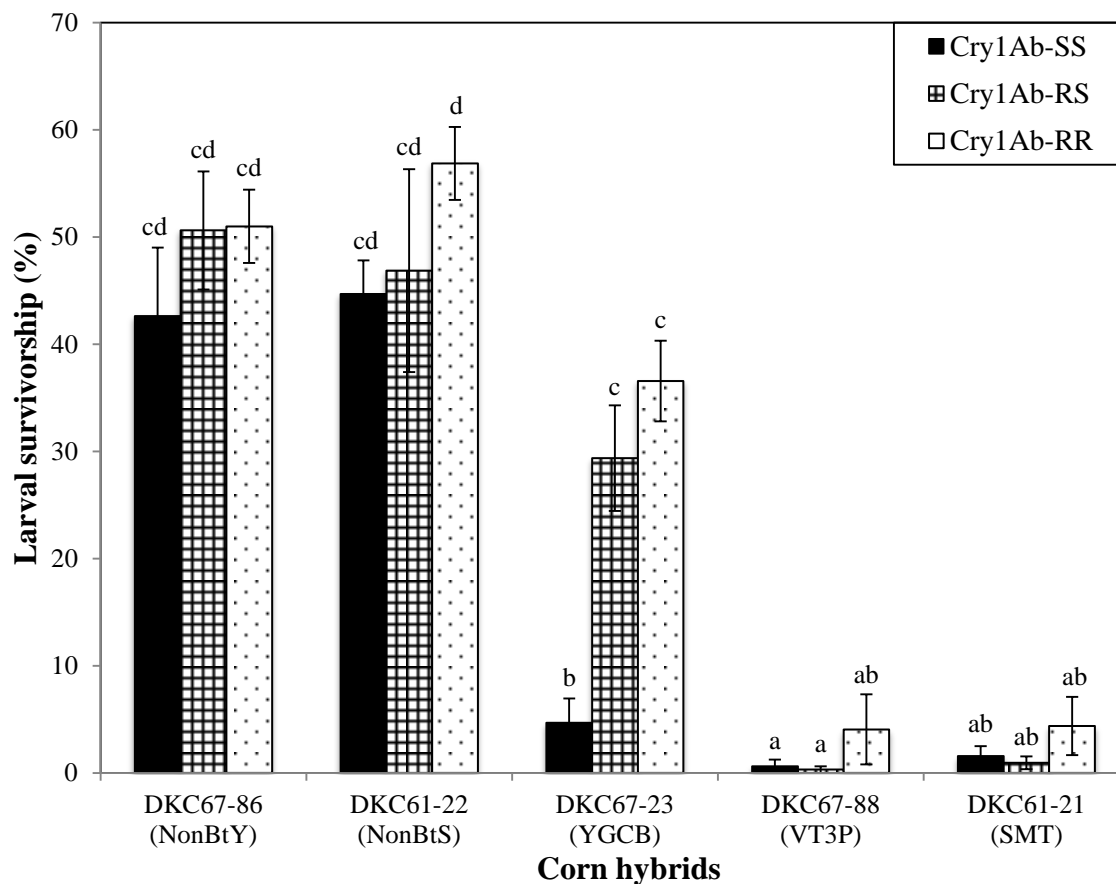
### **2.3.2. Larval Survival and Plant Injury of Cry1Ab-SS, -RS, and -RR Genotypes of *D. saccharalis* on Intact Plants of Two Non-Bt and Three Bt Corn Hybrids: Trial One-2010**

In the first greenhouse trial, which was conducted in 2010, the main effect of corn hybrid on larval survivorship of *D. saccharalis* after 21 days on intact plants was significant ( $F = 111.35$ ;  $df = 4, 42$ ;  $P < 0.0001$ ). The effect of insect genotype and the interaction of corn hybrid and insect genotype was also significant ( $F = 11.43$ ;  $df = 2, 42$ ;  $P = 0.0001$  for insect genotype and  $F = 2.76$ ;  $df = 8, 42$ ;  $P = 0.0153$  for interaction). Larval survivorship on the two non-Bt corn hybrids ranged from 42.6 to 56.9% and was not significantly ( $P > 0.05$ ) different across the three insect genotypes (Fig. 2.2). Cry1Ab-SS was susceptible to YieldGard® corn plants with only a 4.7% survivorship after 21 days. Both Cry1Ab-RR and Cry1Ab-RS larvae survived well on Cry1Ab corn plants with a 21-days survivorship of 36.6 and 29.4%, respectively. The survivorship rates of Cry1Ab-RR and -RS larvae observed on YieldGard® plants was generally not significantly ( $P < 0.05$ ) different from those recorded on non-Bt plants.



**Fig. 2.1.** Larval survivorship (% mean  $\pm$  sem) of Cry1Ab-susceptible (Cry1Ab-SS), -heterozygous (Cry1Ab-RS), and -resistant (Cry1Ab-RR) genotypes of *Diatraea saccharalis* after 6 days on leaf tissue of two non-Bt and three Bt corn hybrids containing single or multiple Cry proteins. Mean values followed by the same letter are not significantly different ( $P < 0.05$ ; LSMEANS test)

As observed in the leaf tissue tests, both pyramided Bt corn hybrids were very effective against all the three insect genotypes. The survivorship rates of Cry1Ab-RR and -RS larvae observed on YieldGard® plants was generally not significantly ( $P < 0.05$ ) different from those recorded on non-Bt plants. As observed in the leaf tissue tests, both pyramided Bt corn hybrids were very effective against all the three insect genotypes. Larval survivorship of the three insect genotypes on the two pyramided Bt corn hybrids was low,  $< 5\%$ , and there were no significant differences among the three insect genotypes and between the two corn hybrids (Fig. 2.2).



**Fig. 2.2.** Larval survivorship (% mean  $\pm$  sem) of Cry1Ab-susceptible (Cry1Ab-SS), -heterozygous (Cry1Ab-RS), and -resistant (Cry1Ab-RR) genotypes of *Diatraea saccharalis* after 21 days on intact plants of two non-Bt and three Bt corn hybrids containing single or multiple Cry proteins (First trial-2010). Mean values followed by the same letter are not significantly different ( $P < 0.05$ ; LSMEANS test)

The number of entry/exit holes counted on stalks also differed significantly among corn hybrids ( $F = 166.21$ ;  $df = 4, 42$ ;  $P < 0.0001$ ), insect genotypes ( $F = 13.08$ ;  $df = 2, 42$ ;  $P < 0.0001$ ), and their interaction ( $F = 3.32$ ;  $df = 8, 42$ ;  $P = 0.0049$ ). The number of entry/exit holes did not significantly differ ( $P < 0.05$ ) between the two non-Bt corn hybrids across all the insect genotypes with an average of 17.9 holes/ stalk (Fig. 2.3a). Cry1Ab-SS larvae on YieldGard® plants made significantly fewer holes on the stalks with an average of 2.1 holes/ stalk. In contrast, Cry1Ab-RR and -RS larvae on YieldGard® plants caused an average of 11 and 10.2

holes/ stalk respectively. The number of holes produced by Cry1Ab-RR on YieldGard® plants was not significantly different from those of the three insect genotypes on the two non-Bt corn hybrids. The difference in the number of holes on YieldGard® plants was also not significant between Cry1Ab-RS and Cry1Ab-RR larvae. As observed in larval survivorship, both pyramided Bt corn hybrids were very effective in reducing stalk boring of *D. saccharalis* regardless of the insect genotypes. Number of holes bored by the three insect genotypes was low, ranged from 0.1-1.2 holes/stalk, and there were no significant differences between the two pyramided Bt corn hybrids and among the three insect genotypes (Fig. 2.3a).

Stalk tunnel length caused by *D. saccharalis* after 21 days was also significantly different among corn hybrids ( $F = 186.59$ ;  $df = 4, 42$ ;  $P < 0.0001$ ), insect genotypes ( $F = 9.02$ ;  $df = 2, 42$ ;  $P = 0.0006$ ), and the interactions of corn hybrids and insect genotypes ( $F = 3.5$ ;  $df = 8, 42$ ;  $P = 0.0035$ ). Stalk tunnel length by the three insect genotypes on the five corn hybrids was highly correlated to the larval survivorship and the number of entry/exit holes. The tunnel length on two non-Bt corn hybrids was not significantly different ( $P > 0.05$ ) and ranged from 46.1 to 70.3 cm/stalk across the three insect genotypes. Cry1Ab-SS larvae on YieldGard® plants caused an average of 6.6 cm tunnel length per stalk, which was significantly shorter ( $P < 0.05$ ) than those observed on non-Bt plants. Both Cry1Ab-RR and -RS larvae caused a significant stalk injury on YieldGard® plants with an average tunnel length of 28.3 and 32.1 cm/stalk, respectively (Fig. 2.3b). The tunnel length on YieldGard® caused by Cry1Ab-RS was not significantly different from those observed on the non-Bt plants, and the tunnel length made by Cry1Ab-RR was also not significantly different from those observed on the non-Bt corn plants infested with Cry1Ab-SS larvae. However, both pyramided Bt corn hybrids were highly effective in reducing stalk tunneling of *D. saccharalis* regardless of the insect genotypes. Tunnel length per stalk on the two

pyramided Bt corn hybrids ranged from only 0.1 to 2.7 cm across the three insect genotypes, which was even significantly shorter than that (6.6 cm) of Cry1Ab-SS on YieldGard® plants. The tunnel length (2.7 cm) of Cry1Ab-RR on Genuity® VT Triple Pro™ hybrid was statistically significantly greater than those (0.1-0.2 cm) of Cry1Ab-RS larvae on the two pyramided Bt corn hybrids, but the differences were small (Fig. 2.3b).

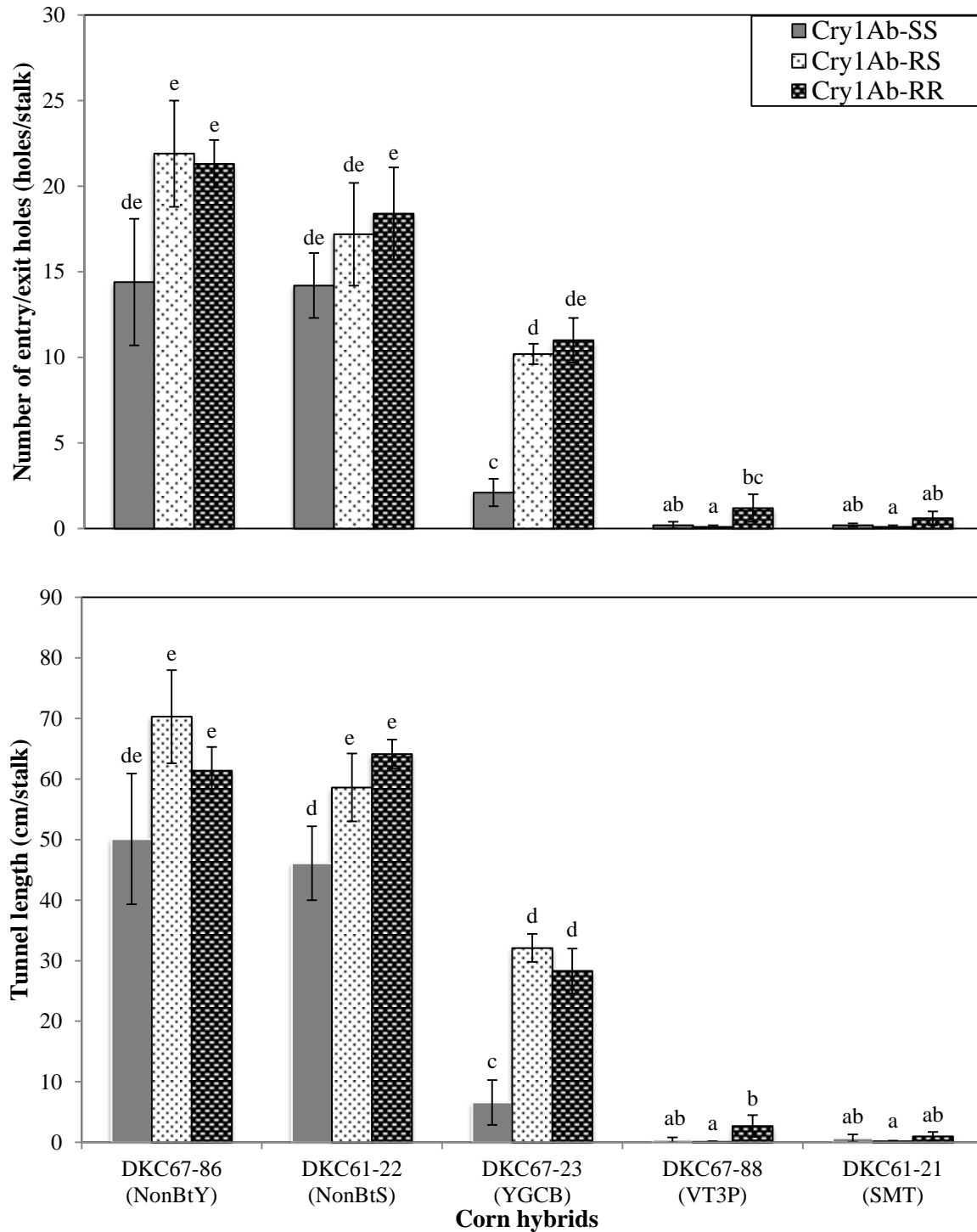
### **2.3.3. Larval Survival and Plant Injury of Cry1Ab-SS, -RS, and -RR Genotypes of *D. saccharalis* on Intact Plants of Two Non-Bt and Three Bt Corn Hybrids: Trial Two-2011**

The overall performance of the three genotypes of *D. saccharalis* on the five corn hybrids was consistent in the two trials conducted in 2010 and 2011. As observed in the trial conducted in 2010, larval survival of *D. saccharalis* after 21 days in the trial performed in 2011 was also significantly affected by corn hybrid ( $F = 194.98$ ;  $df = 4, 42$ ;  $P < 0.0001$ ), insect genotype ( $F = 17.0$ ;  $df = 2, 42$ ;  $P < 0.0001$ ), and their interaction ( $F = 5.01$ ;  $df = 8, 42$ ;  $P = 0.0002$ ).

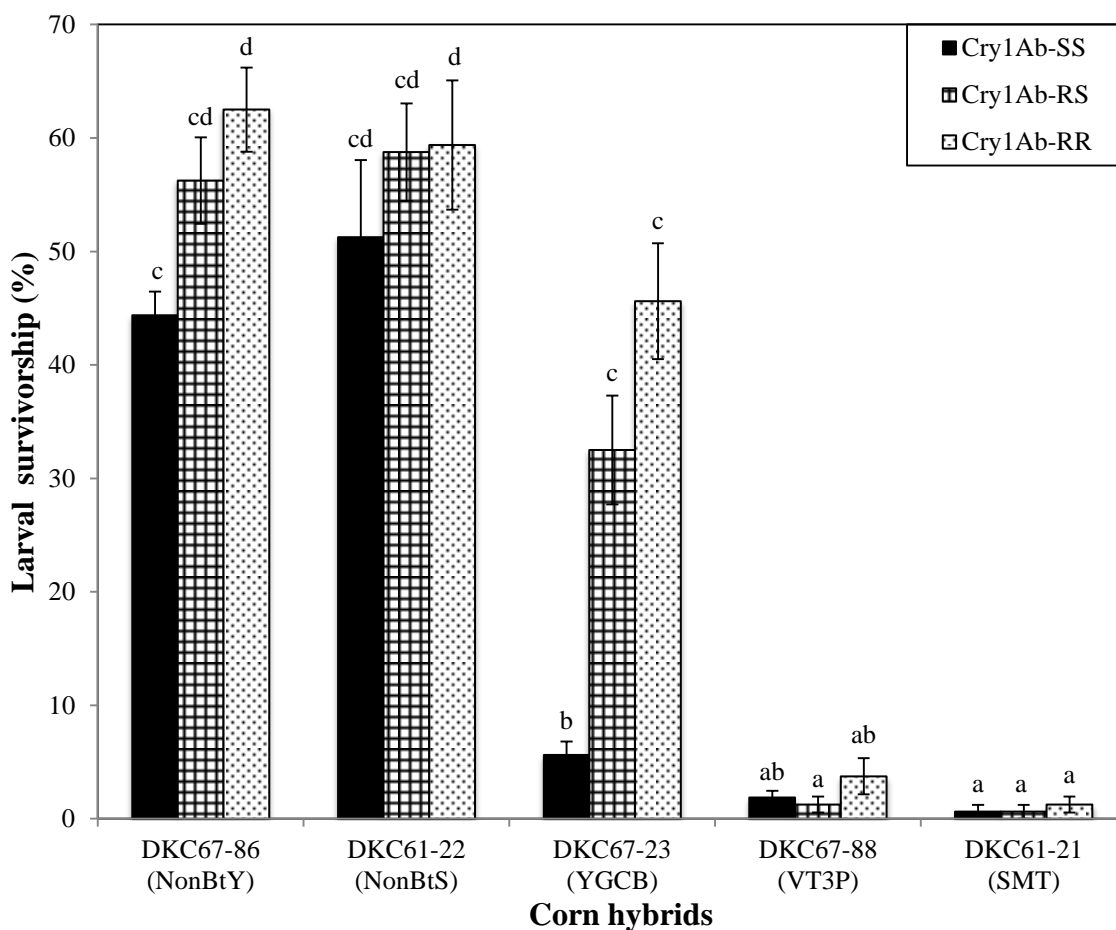
Survivorship on the two non-Bt corn hybrids ranged from 43.4 to 62.5% and was not significantly different ( $P > 0.05$ ) across the three insect genotypes. Survivorship of Cry1Ab-RR and -RS on YieldGard® plants was 45.6 and 32.5%, respectively, which was significantly greater than that (5.6%) of Cry1Ab-SS but was not significantly different from most of those observed on the two non-Bt plants. Again, both pyramided Bt corn hybrids were very effective against all three insect genotypes. Larval survivorship on the two pyramided Bt corn hybrids ranged from 0.6 to 3.8% and was not significantly different between the two hybrids and across the three insect genotypes (Fig. 2.4).

Data on number of entry/exit holes recorded in the 2011 trial were also consistent with those observed in the 2010 trial. The main effect of corn hybrid and insect genotype on number of entry/exit holes was significant ( $F = 337.06$ ;  $df = 4, 42$ ;  $P < 0.0001$  for corn hybrid and





**Fig.2.3.** Number of entry/exit holes (mean  $\pm$  sem) and stalk tunnel length (cm, mean  $\pm$  sem) of Cry1Ab-susceptible (Cry1Ab-SS), -heterozygous (Cry1Ab-RS), and -resistant (Cry1Ab-RR) genotypes of *Diatraea saccharalis* after 21 days on intact plant of two non-Bt and three Bt corn hybrids containing single or multiple Cry proteins (First trial- 2010). Mean values followed by the same letter are not significantly different ( $P < 0.05$ ; LSMEANS test)



**Fig. 2.4.** Larval survivorship (% mean  $\pm$  sem) of Cry1Ab-susceptible (Cry1Ab-SS), -heterozygous (Cry1Ab-RS), and -resistant (Cry1Ab-RR) genotypes of *Diatraea saccharalis* after 21 days on intact plants of two non-Bt and three Bt corn hybrids containing single or multiple Cry proteins (Second trial-2011). Mean values followed by the same letter are not significantly different ( $P < 0.05$ ; LSMEANS test)

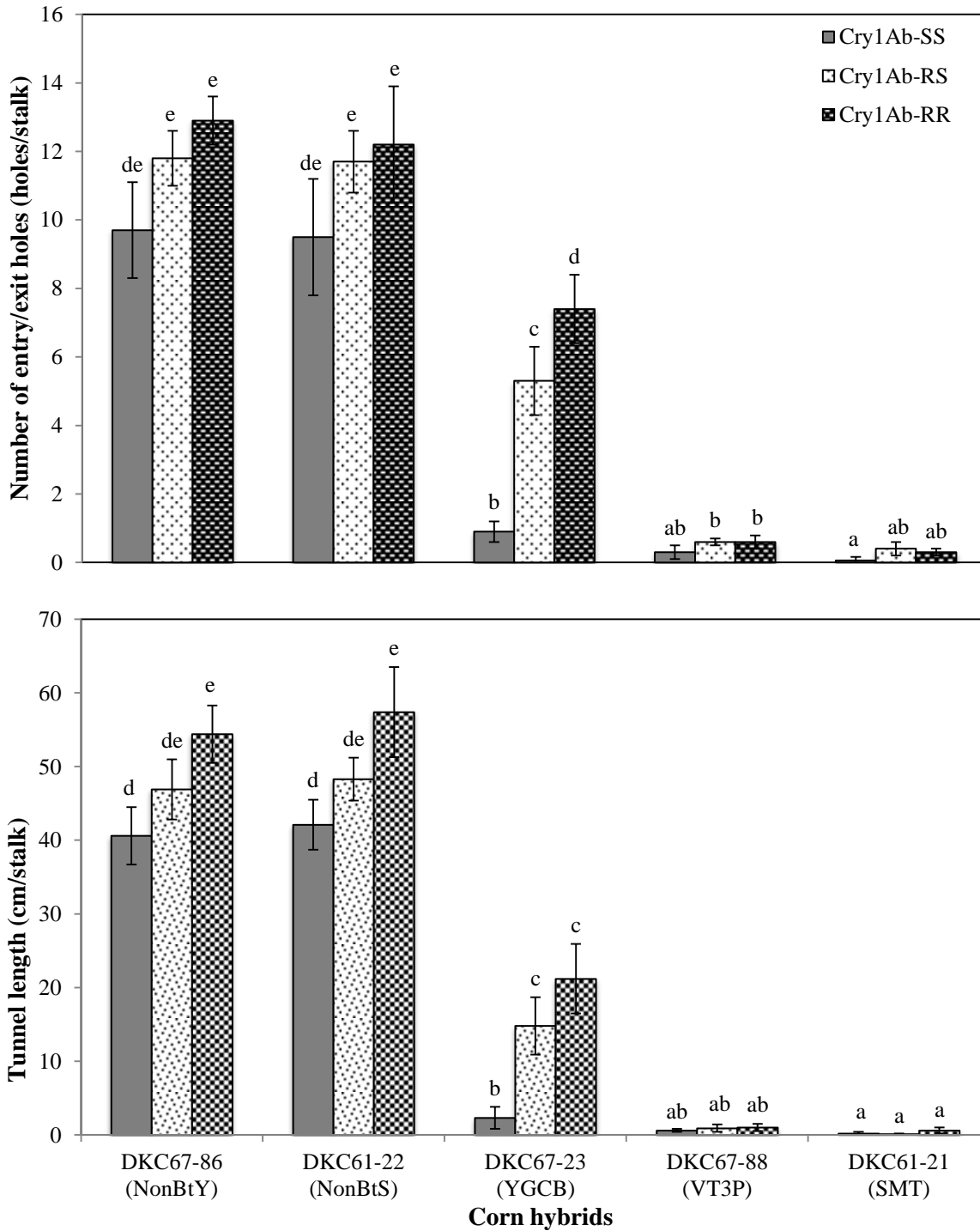
$F = 31.22$ ;  $df = 2, 42$ ;  $P < 0.0001$  for insect genotype). The interaction of corn hybrid and insect genotype was also significant ( $F = 8.39$ ;  $df = 8, 42$ ;  $P < 0.0001$ ). Number of entry/exit holes on non-Bt corn plants ranged from 9.5 to 12.9 and the number was not significantly different between the two hybrids and among the three insect genotypes (Fig. 2.5a). An average of 7.5 and 5.3 holes/ stalk were observed on YieldGard<sup>®</sup> plants that were infested with Cry1Ab-RR and Cry1Ab-RS, respectively. The number of holes on YieldGard<sup>®</sup> plants caused by

Cry1Ab-RR or Cry1Ab-RS was significantly less than those observed on the two non-Bt corn plants but was significantly greater than those (0.9 holes/stalk) made by Cry1Ab-SS larvae. Again, both pyramided Bt corn hybrids were effective in reducing the number of holes caused by *D. saccharalis* regardless of the insect genotype. Number of entry/exit holes on the two pyramided corn hybrids was <1 per stalk and was in general not significantly different between the two corn hybrids and among the insect genotypes (Fig. 2.5a).

As observed in the first trial, the tunnel length in stalks of the five corn hybrids across the three insect genotypes was highly correlated with the larval survival and number of entry and exit holes on the stalks. Tunnel length differed significantly among corn hybrids ( $F = 250.28$ ;  $df = 4, 42$ ;  $P < 0.0001$ ), insect genotypes ( $F = 13.38$ ;  $df = 2, 42$ ;  $P < 0.0001$ ), and their interaction ( $F = 5.46$ ;  $df = 8, 42$ ;  $P < 0.0001$ ). Tunnel length on the two non-Bt corn hybrids ranged from 40.6 to 57.4 cm/stalk and was not significantly different ( $P > 0.05$ ) across the three insect genotypes. Larvae of Cry1Ab-RR and -RS on YieldGard<sup>®</sup> plants caused an average tunnel length of 21.2 and 14.8 cm/stalk, respectively, which was significantly ( $P < 0.05$ ) shorter than those of the three insect genotypes on non-Bt plants but was significantly ( $P < 0.05$ ) longer than that (2.3 cm/stalk) of Cry1Ab-SS on YieldGard<sup>®</sup> plants. In contrast, larvae of *D. saccharalis* caused only very short tunnels,  $\leq 1$  cm/stalk, on the two pyramided Bt corn hybrids regardless of the insect genotype (Fig. 2.5b).

#### **2.3.4. Dominance Level ( $D_{ML}$ ) of Cry1Ab Resistance in *D. saccharalis***

Because survival of all three genotypes of *D. saccharalis* was very low on the two pyramided Bt corn hybrids, dominance level,  $D_{ML}$ , of Cry1Ab resistance in *D. saccharalis* was calculated only for the test with the YieldGard<sup>®</sup> Bt hybrid. The  $D_{ML}$  value was 0.5 based on the 6-day larval survivorship on the leaf tissue test and 0.67-0.78 in the intact plant tests in the



**Fig.2.5.** Number of entry/exit holes (mean  $\pm$  sem) and stalk tunnel length (cm, mean  $\pm$  sem) of Cry1Ab-susceptible (Cry1Ab-SS), -heterozygous (Cry1Ab-RS), and -resistant (Cry1Ab-RR) genotypes of *Diatraea saccharalis* after 21 days on intact plants of two non-Bt and three Bt corn hybrids containing single or multiple Cry proteins (Second trial-2011). Mean values followed by the same letter are not significantly different ( $P < 0.05$ ; LSMEANS test).

greenhouse. The results suggested that the Cry1Ab resistance in *D. saccharalis* was functionally incompletely dominant on Cry1Ab corn leaf tissue and intact Cry1Ab corn plants (Table 2.2).

**Table 2.2.** Effective dominance level ( $D_{ML}$ ) of Cry1Ab resistance in *Diatraea saccharalis* on Cry1Ab corn leaf tissue and intact Cry1Ab corn plants

<b>Trial</b>	<b>Corn hybrid</b>	<b><math>D_{ML}</math></b>
Leaf tissue bioassay	YieldGard <sup>®</sup>	0.50
Intact plants in 2010	YieldGard <sup>®</sup>	0.78
Intact plants in 2011	YieldGard <sup>®</sup>	0.67

## 2.4. Discussion

Data on the larval survival and plant injury showed that the three insect genotypes of *D. saccharalis* were equally effective in establishing themselves on the two non-Bt corn plants. All three insect genotypes survived well on non-Bt leaf tissue in the laboratory bioassays and on non-Bt intact plants in the greenhouse tests. The larval survivorship (72-84% on leaf tissue after 6 days and 42.6-62.5% on intact plants after 21 days) observed in the current study was similar to that reported in other earlier studies (Kumar and Mihm, 1996; Walker et al., 2000; McAllister et al., 2004; Wu et al., 2007; Ghimire et al., 2011). Larvae of all three genotypes of *D. saccharalis* on non-Bt corn plants also made a substantial number of entry/exit holes on the stalks and caused significant tunnel length inside stalks. The results suggest that the artificial diet and leaf tissue selection had not measurably reduced their adaptation to corn plants. As reported in two previous studies (Wu et al., 2007; Ghimire et al., 2011), larvae of the Cry1Ab resistant genotype of *D. saccharalis* in the current study demonstrated a high survivorship on both leaf tissue and intact plants of YieldGard<sup>®</sup> plants expressing the Cry1Ab protein. The results again confirmed that the Cry1Ab-RR genotype of *D. saccharalis* was highly resistant to the Cry1Ab

corn plants.

To delay resistance development, a “high dose/structured refuge” strategy has been adopted for planting the first generation Bt corn that expresses a single Bt protein (e.g. YieldGard<sup>®</sup> Bt corn). One of the key assumptions for the “high dose/refuge” strategy is that resistance in the target species should be recessive so that at least 95% resistant heterozygotes can be killed by “high dose” expressed Bt corn (Andow & Hutchison, 1998; USEPA, 2001; Bourguet et al., 2003). However, both the leaf tissue bioassays and intact plant tests showed a significant survivorship of the Cry1Ab-RS genotype. In both greenhouse trials, Cry1Ab-RS larvae on intact Cry1Ab plants demonstrated a similar ( $P>0.05$ ) survivorship to that of the Cry1Ab-RR larvae. Tunnel length inside the stalks of Cry1Ab corn plants caused by Cry1Ab-RS larvae in both trials was also not significantly different compared to that caused by Cry1Ab-RR. These results suggested that the Cry1Ab resistance in *D. saccharalis*, rather than recessive, was functionally incompletely dominant for the Cry1Ab corn hybrid tested in this study. The effective dominance levels estimated using the method described in Bourguet et al. (2000) was 0.50 based on the leaf tissue bioassay and 0.67-0.78 based on the survival observed on the intact plant tests. In other words, the results of this study showed that the Cry1Ab corn hybrid did not express a “high dose” as defined in the “high dose/refuge” strategy for *D. saccharalis*. Several other Cry1Ab corn hybrids evaluated in two previous studies (Wu et al., 2007; Ghimire et al., 2011) also did not provide a high dose for *D. saccharalis*, especially in the reproductive plant stages.

Other previous studies showed that Cry1Ab resistance in *D. saccharalis* was not associated with any fitness costs (Wu et al., 2009) and the resistance was very stable (Huang et al., 2011b, c). Laboratory bioassays showed that resistance level to purified Cry1Ab protein in

Cry1Ab-RR strain did not decrease after 24 generations without selection. In addition, a 6-year resistance monitoring showed that resistance allele frequency in Louisiana populations of *D. saccharalis* to Cry1Ab corn was low from 2004-2008 with a combined frequency of 0.0011 and a 95% CI of 0.0003 to 0.0024 (Huang et al., 2012). However, the resistance allele frequency in the populations collected during 2009 increased significantly, reached 0.16, which was 14 times greater than that of the populations sampled during 2004-2008 (Huang et al., 2012). Together with previous data, the results suggest that, compared to other corn stalk boring pests such as *O. nubilalis* or *D. grandiosella*, *D. saccharalis* appears to have a higher risk for resistance development if Cry1Ab corn continues to be widely used in the U.S. mid-south region (Stodola et al., 2006; Yue et al., 2008; Tan et al., 2011; Huang et al., 2012).

In spite of the high resistance to Cry1Ab corn, both Cry1Ab-RR and -RS larvae showed 100% mortality after 6 days on leaf tissue of the two pyramided Bt corn hybrids. Performance of the two pyramided Bt corn hybrids in the greenhouse tests also showed high effectiveness against all the three genotypes of *D. saccharalis* with a 21-day larval survivorship of <5% and <3.75% for the 2010 and 2011 trials, respectively. The limited larval survivorship and plant injury (both entry/exit holes and tunnel length) on the two pyramided corn hybrids were similar among the three insect genotypes. The results suggested that the highly resistant (Cry1Ab-RR) strain of *D. saccharalis* was susceptible to both the pyramided Bt corn hybrids. Although data generated for this study could not provide sufficient information to determine if the two pyramided Bt corn hybrids produced a “high dose” of Bt proteins for *D. saccharalis* as defined in the “high dose/refuge” strategy, the results of this study provided clear evidence that the novel pyramided Bt corn hybrids are effective against *D. saccharalis* and can overcome the Cry1Ab resistance in *D. saccharalis*. Both Genuity® VT Triple PRO™ and Genuity® SmartStax™ corn

expresses the Cry1A.105 and Cry2Ab2 proteins. The Cry1A.105 is a chimeric gene comprising of domains I and II which are identical with the respective domains from Cry1Ab and Cry1Ac and domain III of Cry1F (Biosafety Clearing-House, 2009). Previous laboratory bioassays showed that the Cry1Ab-resistant strain of *D. saccharalis* demonstrated only a very low level (4.1-fold) of resistance to the Cry1A.105 protein and was equally susceptible to the Cry2Ab2 protein as its Cry1Ab-SS counterpart (Wu et al., 2009). Additionally, laboratory and greenhouse tests with two experimental corn lines expressing Cry1A.105 and Cry2Ab2 proteins also showed that the pyramided Bt corn lines could completely overcome the Cry1Ab-resistance in *D. saccharalis* (Ghimire et al., 2011). Results of the previous and current studies showed that the pyramided corn technologies expressing Cry1A.105 and Cry2Ab2 should provide a means for managing the Cry1Ab resistance in *D. saccharalis*.

Furthermore, a previous study, using an F<sub>2</sub> screen method, examined 735 feral individuals of *D. saccharalis* collected from multiple locations in Louisiana and Mississippi during 2008 and 2009 for resistance to MON 89034, Genuity® VT Triple Pro™ and Genuity® SmartStax™ (Huang et al., 2011a). The F<sub>2</sub> screen did not detect any of these feral individuals of *D. saccharalis* possessing joint resistance alleles to the three pyramided Bt corn technologies. The MON 89034 corn used in the F<sub>2</sub> screen also contained Cry1A.105 and Cry2Ab2 genes. The results suggest that (joint) resistance alleles to these pyramided Bt corn technologies are rare (Huang et al., 2011a, b).

Genuity® VT Triple PRO™ and Genuity® SmartStax™ corn are the first two commercially available pyramided Bt corn technologies targeting above-ground lepidopteran species in the United States. At the same time, another pyramided Bt corn technology, Genuity® VT Double PRO™ which also contains both Cry1A.105 and Cry2Ab2 genes, has also recently



become commercially available in the United States. Since 2010, with the availability of these pyramided Bt corn technologies, area planted with the single gene Cry1Ab corn in the U.S. mid-south region has been reduced significantly. The majority of the currently planted Bt corn in the U.S. mid southern region contains pyramided Bt genes. The single-gene Cry1Ab corn is expected to be completely replaced by the pyramided Bt corn in the near future. Results of current study, together with the previous data, support the use of pyramided Bt corn for managing *D. saccharalis* in the mid-southern region of the United States. Despite the detection of a significant increase in resistance allele frequency to Cry1Ab corn in 2009, the timely switching from single-gene Cry1Ab corn to pyramided Bt corn should prevent further increases in Cry1Ab resistance allele frequency and thus ensure the continued success of Bt corn for managing *D. saccharalis* in the U.S. mid-south region.

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## **CHAPTER 3: OCCURRENCE AND LARVAL MOVEMENT OF SUGARCANE BORER *DIATRAEA SACCHARALIS* (F.) (LEPIDOPTERA: CRAMBIDAE) IN DIFFERENT PLANTING PATTERNS OF NON-BT AND BT CORN CONTAINING PYRAMIDED TRAITS**

### **3.1. Introduction**

Over the years, a “high dose/structured refuge” strategy has been the primary insecticide resistance management (IRM) strategy for planting Bt corn in the United States. This strategy involves planting a majority of corn in an area (e.g. a farm) with high dose Bt corn that can kill the individuals carrying only one copy of resistant genes (USEPA, 2001). The remaining area is planted to non-Bt varieties that serve as a refuge for Bt-susceptible insects. The strategy takes advantage of insect movement between Bt and non-Bt refuge fields such that the rare resistant survivors from Bt plants and susceptible insects from the non-Bt refuge plants can mate randomly. Therefore, majority of the offspring carrying resistance alleles should be heterozygous and thus should be killed by “high dose” Bt corn plants. As a result, resistance allele frequency in field populations of the target species can be maintained at low levels for long period of time (USEPA, 2001; Qureshi et al., 2006).

In the case of “structured refuge” planting of Bt corn targeting above-ground lepidopteran pests, in the U.S. outside the cotton-producing regions, requirements call for planting 20% (for single-gene expressed Bt corn) or 5% (for pyramided Bt corn) refuge of non-Bt corn on every farm that plants Bt corn, while in the maize-cotton overlapping regions, a minimum of 50% (for single-gene Bt corn) or 20% (for pyramided Bt corn) non-Bt refuge corn is required (Monsanto, 2012; Pioneer, 2011). Refuge plants in the structured refuge strategy are to be within 800 m of the Bt corn field in each farm (USEPA, 2001; Monsanto, 2010). Producer compliance for the structured refuge requirement has always been a problem. During the earlier years of

commercialization of Bt crops, a relatively high rate of compliance (e.g. 86-92%) for the refuge requirement was reported for U.S. Bt crop growers (AGBSTC, 2005; USEPA, 2010).

Unfortunately, compliance rates dropped to 74-80% in 2007 and 2008. Similar declining trend in structured refuge planting was also reported in Canada. The compliance to structured refuge slipped from 85% in 2003 to 61% in 2009 (Dunlop, 2009).

During the 2010-2011 crop seasons, transgenic corn technologies (e.g. Genuity<sup>®</sup> SmartStax<sup>™</sup>, Agrisure<sup>®</sup> Viptera<sup>™</sup> 3111) expressing more than one dissimilar Bt protein that target lepidopteran pests were first commercially planted in the United States. The use of pyramided Bt corn hybrids is expected to delay resistance development in target insect populations. Because of compliance issues in the use of the “structured refuge” IRM strategy, the U.S. EPA approved a seed mixture refuge strategy (also called “refuge-in-the-bag” or “RIB”) for planting pyramided Bt corn hybrids in the north U.S. Corn Belt where no cotton is planted (Monsanto, 2011). For the “RIB” strategy, a portion of non-Bt corn seeds is mixed with Bt corn seeds in each bag by seed industries prior to being sold to farmers. Farmers just need to buy the premixed seeds and plant in their fields (Monsanto, 2011). Therefore, compliance by farmers to the refuge requirement will no longer be an issue. In structured refuge, the dispersal of adults is essential (Gould, 1994., Ostlie., 1997; Hellmich et al., 1999; Shelton et al., 2000; Qureshi et al., 2006) but for the sake of “RIB” strategy it is the larvae that matter. Therefore, the major concern related to the use of the “RIB” strategy is larval movement among Bt and non-Bt plants which may create a more favorable environment for resistance development in target pest populations. For example, movement of susceptible larvae from non-Bt refuge plants to Bt plants in RIB strategy could cause a greater mortality to susceptible insects than in structured refuge planting and thus result in a lower refuge population (Davis and Onstad, 2000). On the other hand,



heterozygous resistant individuals or those insects containing minor resistance alleles could feed on non-Bt plants first and later move to Bt plants and survive because late-instars of corn borers are much less susceptible to Bt toxins (Huang et al., 1999; Walker et al., 2000; Huang et al., 2006). Therefore, the differential susceptibility among instars and larval movement among Bt and non-Bt plants could create a sub-lethal dose exposure of target pests and promote build-up of resistance in target pest populations by increasing survival of heterozygotes. Furthermore, pollen contamination from Bt to non-Bt plants may also create sub-lethal exposures in fields having non-Bt plants planted in close proximity with Bt plants leading to cross pollination (Burkness, 2011). For these reasons, the seed mixture refuge strategy was not considered an appropriate IRM strategy for single-gene Bt corn, although it was also discussed as a potential strategy prior to the commercial use of Bt corn (USEPA, 2001). A few models have shown that “RIB” could be an effective IRM strategy for planting pyramided Bt corn (GH in press). However, field data to support the “RIB” strategy for the pyramided Bt corn are still very limited (Alyokhin, 2011; Onstad et al., 2011).

The sugarcane borer, *Diatraea saccharalis* (F.), is a dominant corn stalk borer in the mid-southern U. S., Caribbean, Central America and warmer parts of South America to Argentina (Capinera, 2001). Since 1999, use of Bt corn has been a primary tool for managing this species on field corn in the U.S. mid-southern region (Castro et al., 2004, Huang et al., 2006). To date, the “RIB” strategy has not been approved in the U.S. southern regions where cotton is also planted. Besides the larval movement issue discussed above, other major concerns for use of “RIB” in the southern region may include: 1) the new Bt corn hybrids may not produce a high dose for the more Bt-tolerant pest species in the south (e.g. corn earworm, fall armyworm); 2) some kernels of refuge plants may also express a low level of Cry proteins due to pollen

contamination of the mixed plantings of Bt and non-Bt corn, which may kill the susceptible refuge insects, especially for the corn earworm which mainly feed on the ears; 3) some proteins of pyramided Bt corn are also expressed in Bt cotton and some targets are major pests for both corn and cotton in the south region (e.g. corn earworm). The objectives of this study were to investigate occurrence and larval movement of *D. saccharalis* in different mixed planting patterns of non-Bt and Bt plants containing pyramided Bt genes and to determine if refuge plants in the “RIB” strategy could provide a similar refuge population of *D. saccharalis* as the “structured refuge” planting. The results should provide valuable information to assess if seed mixtures could be an appropriate refuge strategy for management of *D. saccharalis* with pyramided Bt corn technologies.

## **3.2. Materials and Methods**

### **3.2.1. Insect Sources**

*D. saccharalis* were obtained from the Corn and Small Grain Insect Research Laboratory in the Department of Entomology, Louisiana State University Agricultural Center (LSU AgCenter) in Baton Rouge, LA. Eggs were produced by a Cry1Ab-susceptible strain (Cry1Ab-SS) of *D. saccharalis* that was established from larvae collected from research fields at the Louisiana State University AgCenter’s Macon Ridge Research Station in Winnsboro, LA during 2009 (32° 8’ 6’’N, 91° 41’ 18’’) (Huang et al., 2011a, b). Larvae of the Cry1Ab-SS strain were reared individually in 30 ml plastic cups (Fill-Rite, Newark, NJ) containing a meridic diet (Bio-Serv, Frenchtown, NJ) until the pupal stage as described in Huang et al. (2006). Pupae were then transferred from the plastic cups to 3.785-liter cardboard cartons (Neptune Paper Products, Newark, NJ) containing approximately 100 g of vermiculite (Sun Gro, Pine Bluff, AR) to allow the adults to mate and oviposit eggs. Each container was lined with a wax paper (Reynolds

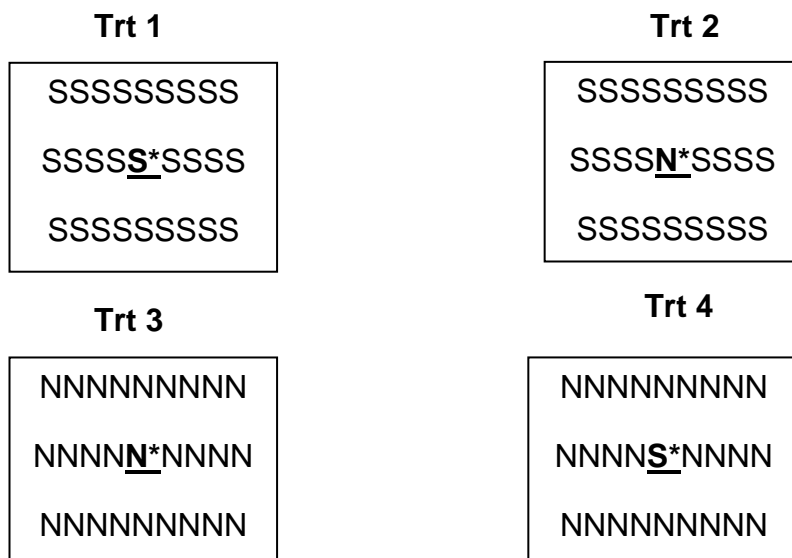
consumer products) for holding the eggs.

### 3.2.2. Corn Hybrids

A Genuity<sup>®</sup> SmartStax<sup>™</sup> hybrid (NF5358QQR) and its genetically closely related non-Bt corn hybrid (NF5358HTT1) used in this study were obtained from Monsanto Company (St. Louis, MO). The Genuity<sup>®</sup> SmartStax<sup>™</sup> hybrid contained six Bt genes including Cry1A.105, Cry2Ab2, and Cry1F for controlling above-ground pests and Cry3Bb1, Cry34Ab1, and Cry35Ab1 for managing below-ground corn rootworms as well as two herbicide resistance traits glyphosate (*Roundup*) and glufosinate-ammonium (*Liberty*) (Gatehouse, 2008). The non-Bt corn expressed both herbicide resistance traits but contained neither of the Bt proteins.

Larval movement, larval occurrence and plant injury of *D. saccharalis* were evaluated in four different planting patterns of Bt and non-Bt plants in greenhouse and open field conditions. Each planting pattern consisted of 3 rows with 9 plants in each row (a total of 27 plants) (Fig. 3.1).

The four different planting patterns (treatments) included: Trt 1) pure stand of 27 SmartStax<sup>™</sup> plants (all Bt); Trt 2) one non-Bt plant in the center surrounded by 26 SmartStax<sup>™</sup> plants (RIB), Trt3) pure stand of 27 SmartStax<sup>™</sup> plants (all NBt) , and Trt 4) one SmartStax<sup>™</sup> plant in the center surrounded by 26 non-Bt plants (C-Bt). The planting pattern of Trt 2 was designed to simulate a 96:4% “RIB”, which was close to the currently used “95:5%” RIB for planting Genuity<sup>®</sup> SmartStax<sup>™</sup> corn in the United States, while Trt 3 was used to simulate a “structured refuge” planting. A total of three different tests were conducted, which included 1) greenhouse evaluation with artificial infestation of eggs on the central plants; 2) open field with artificial infestation of eggs in the central plants; and 3) open field with artificial infestation of larvae on all plants. A randomized complete block design was used for all three tests in the greenhouse and in the open field conditions.



**Fig.3.1.** Four planting patterns of Bt and non-Bt corn used for evaluation of larval movement, larval occurrence, and plant injury of *Diatraea saccharalis* in greenhouse and field trials. N= non-Bt plant, S= SmartStax<sup>TM</sup> plant, N\*= center non-Bt plant, S\*= center SmartStax<sup>TM</sup> plant

### 3.2.3. Greenhouse Evaluations with Artificial Infestation

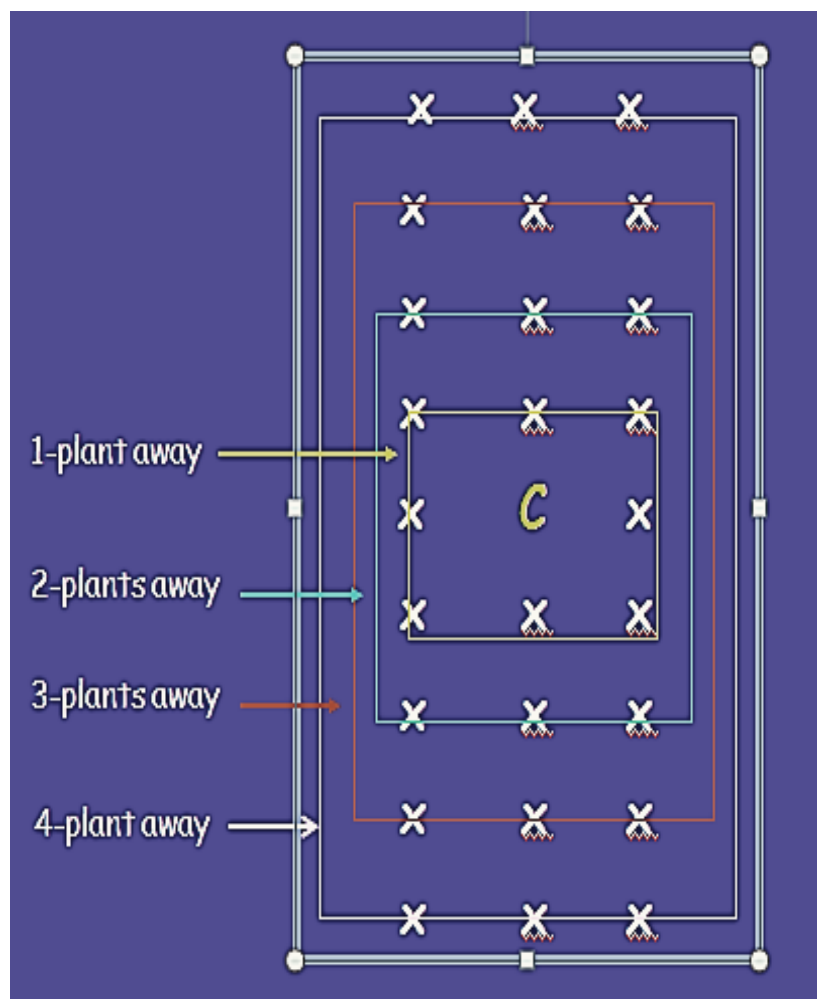
In 2011, one greenhouse test was conducted to investigate the larval movement and plant injury of *D. saccharalis* on the four planting patterns of Bt and non-Bt corn mentioned above. In the trial, seeds of Genuity<sup>®</sup> SmartStax<sup>TM</sup> and the non-Bt corn were planted in 5 gallon plastic pots containing  $\approx$  5 kg of standard potting soil mixture (Perfect Mix<sup>TM</sup>, Expert Gardener products, St. Louis, MO) in a greenhouse at the Louisiana State University Agricultural Center's greenhouse in Baton Rouge, LA as described in Wu et al. (2007). The planting/spacing in the greenhouse was similar to that used in farmer's fields. Two seeds were planted in each pot at approximately 20 cm apart.

The pots were placed on four tables in the greenhouse allowing proper distance from pot to pot and table to table without the pots touching each other within a column. The pots were arranged to allow a distance of 20 cm from the two adjacent plants in the two pots within a row

and  $\approx 60$  cm from one row to the next row on the same table. A mixture of southern turf builder, lawn fertilizer (2% iron, 32N-0P-10K, Scotts Company, OH) and the Lawn and Garden plant food (13N-13P-1K, Meherrin fertilizer, Inc, NC) were applied at V2 and V8 plant growth stages (Ritchie et al., 1993).

Topping -up and irrigation among other management practices were given on need basis to ensure optimum growth. The EnviroLogix, Quantiplate<sup>TM</sup> kits for Cry1Ab/Cry1Ac, Cry 1F, Cry2Ab2, (500 Riverside Industrial Parkway, Portland, ME) were used to confirm the expression of Bt proteins in corn plants before egg infestation. The trials were conducted at reproductive plant stages to simulate the second generation infestations in open field. Plants in each plot were assigned a number and distance between a plant and the central plant was measured. The surrounding plants distributed from the central plant was between 18-43 cm, 36-52 cm, 56-66 cm and 75-86 cm for the 1 plant, 2 plants, 3 plants and 4 plants- away from the central plant (Fig. 3.2). Each center plant in each treatment plot was infested with 50 ready-to-hatch eggs (2 to 3 egg masses) by stapling a piece of wax paper containing the eggs at the abaxial (underside) of a leaf with a visible collar.

The number of un-hatched eggs was checked 2- 3 days after infestation. Egg hatching rates were calculated by subtracting the number of un-hatched eggs from the total number of infested eggs divided by the total number of eggs infested. There were four replications each with a 1- meter distance between replications for each planting pattern in a randomized complete block design. All plants were cut after 21 days using destructive sampling method and examined to record number of live insects and tunnel length inside stalks. Data on number of live insects recovered after 21 days were organized for distance class among the four planting pattern. Distance class 0 referred to the center-infested plants (focal plants), distance class 1 referred to



**Fig. 3.2.** A diagram showing how data on larval occurrence and plant injury of *Diatraea saccharalis* were organized for statistical analysis, C- refers to the center infested plant.

all eight plants that were 1-plant away from the central plant, distance class 2 referred to all six plants that were 2-plants away from the center plant, distance class 3 referred to all six plants that were 3-plants away from the center plant and distance class 4 referred to all the six peripheral plants that were 4-plants away from the center plant (Fig. 3.2). Larval distribution in the five distance classes of a planting pattern was compared to another planting pattern by using a multinomial logistic regression (Multinomial logit) model (Agresti, 2007). The input data used by log-linear models are arranged in a 5 by 4 contingency table format. The number of insects

was categorically distributed over distance classes (Snedecor and Cochran, 1989). The interpretation is based on the odds ratios taking an assumption for proportional odds. The multinomial logit analysis was done using SAS PROC LOGISTIC procedure and the equation for the linear model was:

$$\log \frac{\pi_j}{\pi_B} = \alpha_j + \beta_{j1} \text{Trt. 1} + \beta_{j2} \text{Trt. 2} + \beta_{j3} \text{Trt. 3}$$

Where  $\pi_j$  is odds for insect occurrence in the  $j^{\text{th}}$  distance class considering  $\pi_B$  as the baseline distance class. The likelihood for an insect occurring in the  $j^{\text{th}}$  distance class of the  $i^{\text{th}}$  treatment compared to the likelihood for the same distance class in baseline treatment (Trt. B) was computed as follows;

$$\frac{\text{odds}}{\text{odds}} = \frac{\frac{\pi_{\text{Dis. } j}}{\pi_{\text{Dis. } B}} \text{Trt. } i}{\frac{\pi_{\text{Dis. } j}}{\pi_{\text{Dis. } B}} \text{Trt. } B} = \exp(\beta_{ij})$$

In some cases, observations had non-positive frequencies or weights in the  $\chi^2$  – analysis, the number of insects as well as tunnel length were also analyzed using one-way analysis of variance (ANOVA) to examine the difference among the four planting patterns at each distance class. Stalk tunnel length was presented as tunnel length (cm) per plant. Data on tunnel length for the ANOVA were first transformed to  $\ln(x+1)$  scale. Treatment means were separated using the LSD tests at  $\alpha = 0.05$  level. In addition, interplant and inter-row larval movement of *D. saccharalis* in different planting patterns were also characterized by calculating the percentages of larvae dispersed from central infested plant and infested rows. Percentage data were then transformed to arcsine scale followed by one-way ANOVA (SAS Institute, 2010). Treatment means were separated using the LSD tests at  $\alpha = 0.05$  level. Untransformed data are presented

in the figures and tables.

#### **3.2.4. Open Field with Artificial Infestation of Eggs on the Central Plants**

During 2011, larval movement and plant injury of *D. saccharalis* in the four planting patterns (Fig. 3.1) were investigated in open field conditions with artificial infestation of eggs on the central plants. Corn seeds were planted approximately 3 weeks ahead of the farmer's normal planting date to limit the natural population of *D. saccharalis*. Planting was carried out maintaining a distance of 2 meters between the plots in a treatment. At VT-R1 plant stage (Ritchie et al., 1993), 50 ready-to-hatch eggs of *D. saccharalis* were infested on the center plant of each plot as described above. A randomized complete block design was used with 7 replications for each planting pattern. In order to document the natural occurrence of *D. saccharalis* at the trial site, an additional four plots of pure stand of non-Bt plants were planted in the trial field. Artificial infestation was not performed for these four plots. Larval occurrence and stalk tunnel length were checked at the same time as those plots that were infested with eggs. Data on larval distribution, insect occurrence and tunnel length were analyzed using the same methods as described in the above greenhouse study.

#### **3.2.5. Open Field Tests with Artificial Infestation of Neonates of *D. saccharalis* on All Plants**

In 2011, one field trial was conducted to examine the occurrence and plant injury of *D. saccharalis* in the four planting patterns of Bt and non-Bt plants as shown in Fig. 3.2. Because the natural occurrence of *D. saccharalis* was very low in 2011, artificial infestations were employed in the test. The test was planted on July 5<sup>th</sup>, 2011 and infested with 10 neonates/ plant for all plants on September 28<sup>th</sup>, 2011. After 3 weeks of the infestations, plants were checked to record number of live insects and tunnel length inside the stalks as described above. There were



5 replications for each treatment.

For data analysis, refuge plants in Trt 2 were considered as a separate treatment. Data collected from the non-Bt refuge plants in Trt 2 were separated from those recorded from the Bt plants and were considered as another treatment. Similarly, data recorded on the central Bt plants in Trt 4 were separated from those surrounding non-Bt plants and were considered as another treatment in the statistical analysis. Data on number of live insects and tunnel length inside stalks were subjected to one-way ANOVA (SAS Institute, 2010) to determine differences among treatments. All data for the ANOVA were transformed to  $\ln(x + 1)$  scale. Treatment means were separated using the LSD test at  $\alpha = 0.05$  level. Untransformed data are presented in the tables.

### **3.3. Results**

#### **3.3.1. Greenhouse Evaluations with Artificial Infestation**

##### **3.3.1.1. Larval Distribution of *D. saccharalis* on Different Planting Patterns of Non-Bt and Bt Plants**

Chi-square analysis showed that there was a significant difference in larval distribution between the pure stand of non-Bt planting (Trt 3) and “RIB” planting (Trt 2) ( $\chi^2 = 4.4104$ ,  $df=1$ ,  $P = 0.0357$ ) (Table 3.1). In the pure stand of non-Bt corn planting, 87.1% live larvae moved away from the central plants and survived on the plants at the 1<sup>st</sup> to the 4<sup>th</sup> distance classes and the plants which hosted the most insect individuals were at the 1<sup>st</sup> distance class (18.0 larvae). In contrast, in the “RIB” planting, 65.1% live larvae were found within 1-plant away and the central non-Bt plants harbored the most individuals (6.3 individuals). For the other pairwise comparisons, larval distribution at each of the five distance classes were not significantly different among the four planting patterns ( $\chi^2 \leq 1.3973$ ,  $df=1$ ,  $P \geq 0.2372$ ) (Table 3.1).

**Table 3.1.** Analysis of maximum likelihood estimates in the logistic procedure for larval distribution of *Diatraea saccharalis* in greenhouse tests in 2011. §

Effect	Class	DF	Estimate	SE	CL		$\chi^2$ square	P> Chisq
Trt1 × Trt3	0	1	-15.3412	5208	<0.001	>999.9	0.0000	0.9977
Trt1 × Trt3	1	1	-15.3032	3809.2	<0.001	>999.9	0.0000	0.9968
Trt1 × Trt3	2	1	-15.4572	4936.9	<0.001	>999.9	0.0000	0.9975
Trt1 × Trt3	3	1	-15.5908	5386.7	<0.001	>999.9	0.0000	0.9977
Trt1 × Trt3	4	1	2.2057	1.4441	0.535	153.86	2.333	0.1267
Trt2 × Trt3	0	1	0.7034	0.3349	1.048	3.895	4.4104	0.0357
Trt2 × Trt3	1	1	-0.0123	0.3089	0.539	1.810	0.0016	0.9684
Trt2 × Trt3	2	1	-0.2537	0.3815	0.367	1.639	0.4425	0.5059
Trt2 × Trt3	3	1	-0.3952	0.4048	0.305	1.489	0.9535	0.3288
Trt2 × Trt3	4	1	-0.6984	0.5908	0.156	1.583	1.3973	0.2372
Trt4 × Trt3	0	1	-15.0013	615.4	<0.001	>999.9	0.0006	0.9806
Trt4 × Trt3	1	1	0.5134	0.3071	0.915	3.051	2.7949	0.0946
Trt4 × Trt3	2	1	0.1849	0.3808	0.570	2.538	0.2359	0.6272
Trt4 × Trt3	3	1	-0.2598	0.4413	0.325	1.832	0.3465	0.5561
Trt4 × Trt3	4	1	-0.3398	0.5958	0.221	2.289	0.3252	0.5685

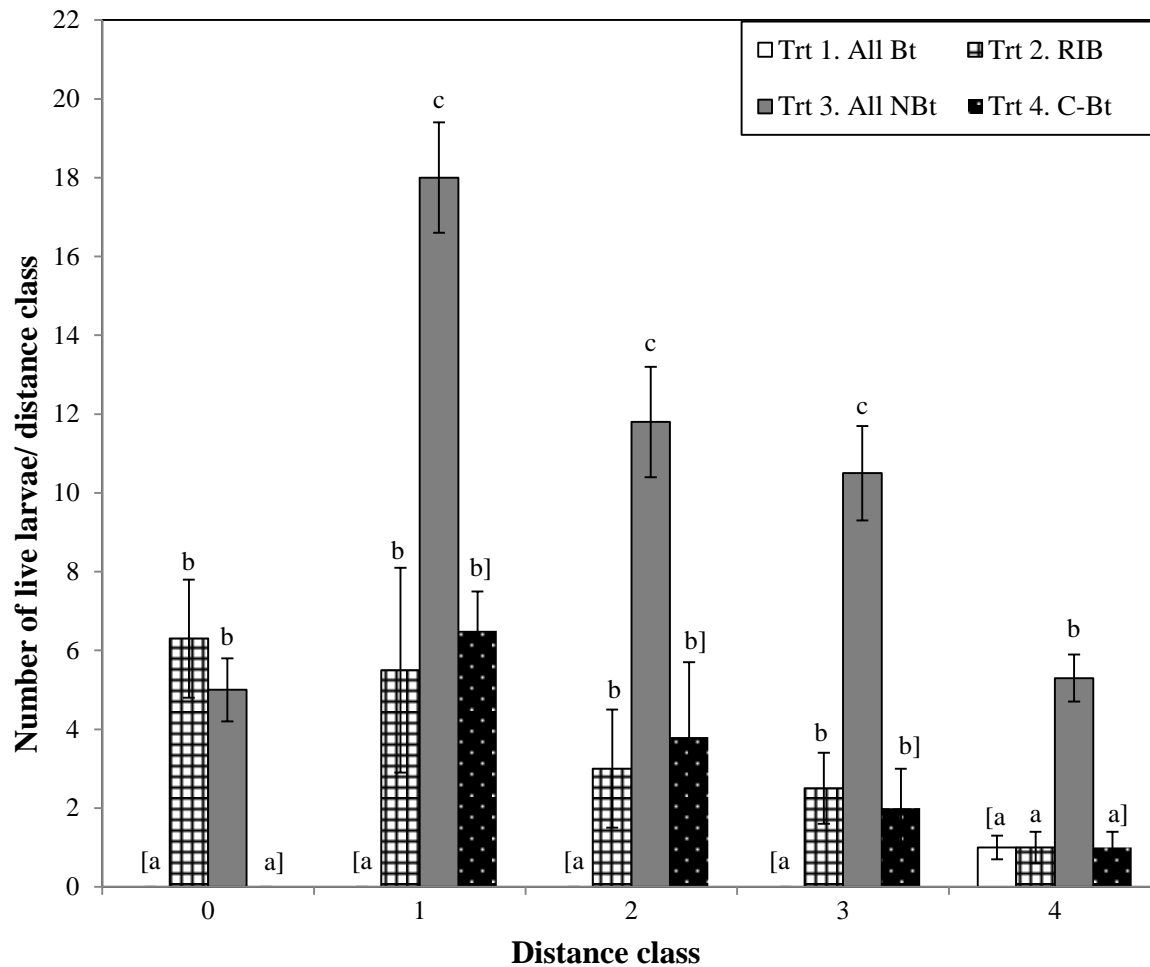
§ Trt 1= pure stand of 27 SmartStax™ plants (All Bt), Trt 2= one non-Bt plant in the center surrounded by 26 SmartStax™ plants (RIB), Trt3= pure stand of 27 non-Bt plants (All NBt), and Trt 4= one SmartStax™ plant in the center surrounded by 26 non-Bt plants (C-Bt).

- Indicates observations having non-positive frequencies or weights.

The table shows distance classes (Class), parameter estimates (Estimates), the corresponding degrees of freedom (DF), Wald's 95% confidence limits (CL), chi-square value and the associated probability of obtaining a larger chi-square value (P> ChiSq).

ANOVA showed that there were significant differences in the number of live insects recovered from the center plants among the four planting patterns ( $F = 39.22$ ;  $df = 3, 9$ ;  $P < 0.0001$ ). After 21 days of egg infestation, an average of 5 live insects were found on the central plants in the pure stand of non-Bt planting, which was similar ( $P > 0.05$ ) to that (6.3 insects) recovered from the central plants in the “RIB” planting. No insects remained in the central plants and survived in the other two planting patterns. Significant differences in number of live insects were also observed among the four planting patterns at each of 1<sup>st</sup> to 4<sup>th</sup> -distance classes ( $F \geq 4.13$ ,  $df = 3, 9$ ;  $P \leq 0.0426$ ). The number of live insects was not significantly different

between “RIB” and Trt 4 at each of the 1<sup>st</sup> to 3<sup>rd</sup> distance classes and no insects survived at these three distance classes in the pure stand of Bt plants (Fig. 3.3). At the 4<sup>th</sup> -distance class, an average of 5.3 live insects were found in the pure stand of non-Bt plants which was significantly greater than that recovered in any other planting patterns.



**Fig. 3.3.** Occurrence of *Diatraea saccharalis* in four planting patterns of Bt and non-Bt corn (mean  $\pm$  sem). Comparisons are made within a distance class among the four planting patterns. Distance class 0 refers to the center infested plants (focal plants); distance class 1 refers to all eight plants that are 1 plant away, distance class 2 refers to all six plants that are 2 plants away, distance class 3 refers to all six plants that are 3 plants, and distance class 4 refers to all six plants that are 4 plants away from the central plant. Mean values followed by a same letter within the same distance class in brackets are not significantly different ( $P < 0.05$ ; LSD test).

### **3.3.1.2. Interplant and Inter-row Movement of *D. saccharalis* in Different Planting Patterns of Non-Bt and Bt Plants**

Because there were no survivors in the pure stand of Bt plants after 21 days of egg infestation, data used in ANOVA for inter-row movement of *D. saccharalis* did not include this planting pattern. Migration off the infested plants and across the infested rows in the greenhouse studies was not significantly different among the planting patterns ( $F=1.96$ ;  $df = 2, 9$ ;  $P=0.1962$ ). However, dispersal from the infested central row to adjacent rows was significantly different ( $F=14.82$ ;  $df = 2,9$ ;  $P<0.0014$ ) among the three planting patterns (Table 3.2). No insects were recovered from the central infested Bt plants in Trt 4. Compared to 92.2% of the survivors that moved away from the central infested plants and survived on surrounding plants in the pure stand of non-Bt plants, a significantly lower ( $P < 0.05$ ) percentage (49.3%) of larvae moved from the center non-Bt plants and survived in the surrounded Bt plants in the “RIB” planting. The percentage (30.5%) of larvae that moved away from the infested row (central row) and survived in the adjacent rows in “RIB” planting was significantly lower ( $P < 0.05$ ) than that (53.5- 56.0%) in the other two planting patterns. There was no significant difference in the percentage of larvae that moved away from the infested rows and survived on the adjacent rows between the pure stand of non-Bt plants (Trt 3) and Trt 4.

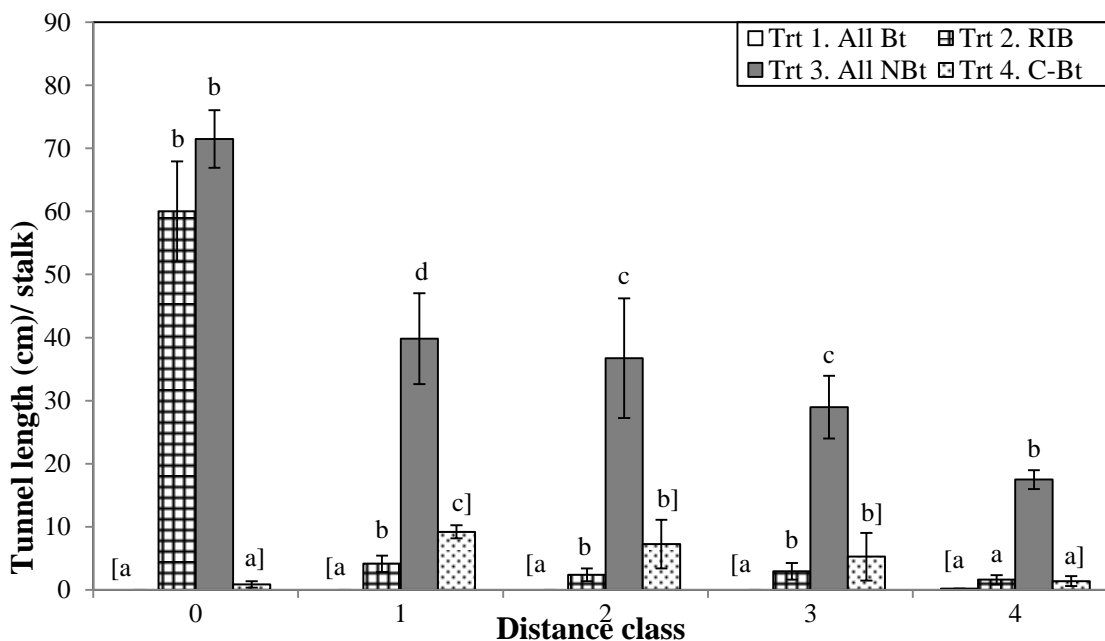
### **3.3.1.3. Plant Injury of *D. saccharalis* in Different Planting Patterns of Non-Bt and Bt Plants**

Tunnel length was significantly different among the four planting patterns for all distance classes ( $F \geq 7.12$ ,  $df=3,9$ ;  $P \leq 0.0095$ ). On the central plants, an average of 71.5 cm tunnel length was observed in the pure stand of non-Bt plants, which was not significantly different than that (60 cm) recorded in the “RIB” planting (Fig. 3.4).

**Table 3.2.** Interplant and inter-row larval dispersal (mean  $\pm$  sem) of *Diatraea saccharalis* in greenhouse study with artificial infestations of 50 ready-to-hatch eggs on center plants in 2011

	Percentage dispersal*	
	Off the central infested plant	Dispersal to adjacent rows
Trt 1. All Bt plants	-	-
Trt 2. RIB	49.3 $\pm$ 19.5 a	30.5 $\pm$ 2.2 a
Trt 3. All Non Bt plants	92.3 $\pm$ 1.4 a	56.0 $\pm$ 2.1 b
Trt 4. Center Bt	100.0 $\pm$ 0.0 a	53.5 $\pm$ 6.0 b
F	1.96	14.82
df	2, 9	2, 9
P- value	0.1962	0.0014

\* Means ( $\pm$  SE) followed with the same letter within a column are not statistically different ( $P < 0.05$ ; LSD test).



**Fig.3.4.** Stalk tunnel length (cm, mean  $\pm$  sem) caused by *Diatraea saccharalis* in four planting patterns of Bt and non-Bt corn. Comparisons are made within a distance class among the four planting patterns. Distance class 0 refers to the center infested plants (focal plants); distance class 1 refers to all eight plants that are 1 plant away, distance class 2 refers to all six plants that are 2 plants away, distance class 3 refers to all six plants that are 3 plants away, and distance class 4 refers to all six plants that are 4 plants away from the central infested plant. Mean values followed by a same letter within the same distance class in brackets are not significantly different ( $P < 0.05$ ; LSD test).

No tunnels were found in the pure stand of Bt corn plants and only very short tunnels (0.86 cm) were located in Trt 4. At the 1<sup>st</sup> to 4<sup>th</sup> distance classes, tunnel length of the pure stand of non-Bt plants was significantly greater than other planting patterns. At distance classes 1, tunnel length of Trt 4 was significantly longer than that of “RIB” planting. Only a short tunnel was observed in the pure stand of Bt corn plants, which was found in plant located at the 4<sup>th</sup> distance class (Fig. 3.4).

### **3.3.2. Open Field with Artificial Infestation of Eggs on the Central Plants**

#### **3.3.2.1 Larval Distribution of *D. saccharalis* on Different Planting Patterns of Non-Bt and Bt Plants**

At the time when data were taken from the trial, no individuals of *D. saccharalis* were found in the four non-Bt plots without artificial infestation. This indicated that natural infestation of *D. saccharalis* at the trial site was low and did not confound with the artificial infestations. Chi-square tests showed that there were no significant differences ( $\chi^2 \leq 0.2.7444$ ,  $df=1$ ,  $P \geq 0.0976$ ) in larval distribution of *D. saccharalis* among the four planting patterns after 21 days of egg infestation (Table 3.3). Further ANOVA showed that the number of live insects recovered among the four planting patterns was significantly different for the center plants and plants in the 1<sup>st</sup> distance classes ( $F \geq 10.02$ ;  $df=3, 18$ ;  $P \leq 0.0004$ ) but not at the greater distances ( $F \leq 1.33$ ;  $df=3, 18$ ;  $P \geq 0.2972$ ). On the central plants, an average of 2.4 live insects was found in the pure stand of non-Bt plants which was similar to that (2.3 insects) observed in “RIB” planting, while no insects were recovered in the central plants in the pure stand of Bt plants and Trt 4. At distance class 1, significantly more insects were found in the pure stand of non-Bt plants than any of the three planting patterns. Some live insects were also located at the 2<sup>nd</sup> to 4<sup>th</sup> distance classes, but in generally the number was small (Fig. 3.5).

**Table 3.3.** Analysis of maximum likelihood estimates in the logistic procedure for larval distribution of *Diatraea saccharalis* in open field plants artificially infested with 50 ready-to-hatch eggs in 2011 studies. §

Effect	Class	DF	Estimate	SE	CL	$\chi^2$	P> Chisq	
Trt1 × Trt3	0	1	-	-	-	-	-	
Trt1 × Trt3	1	1	-	-	-	-	-	
Trt1 × Trt3	2	1	-	-	-	-	-	
Trt1 × Trt3	3	1	-	-	-	-	-	
Trt1 × Trt3	4	1	-	-	-	-	-	
Trt2 × Trt3	0	1	0.7709	0.4654	0.868	5.382	2.7444	0.0976
Trt2 × Trt3	1	1	-16.493	1545.1	<0.001	>999.9	0.0001	0.9915
Trt2 × Trt3	2	1	0.0488	0.8867	0.185	5.970	0.003	0.9561
Trt2 × Trt3	3	1	-16.2661	3185.3	<0.001	>999.9	0.0000	0.9959
Trt2 × Trt3	4	1	-16.5538	6370.6	<0.001	>999.9	0.0000	0.9979
Trt4 × Trt3	0	1	-17.7062	6348.8	<0.001	>999.9	0.0000	0.9978
Trt4 × Trt3	1	1	-0.1335	1.1915	0.085	9.041	0.0126	0.9108
Trt4 × Trt3	2	1	1.0296	1.2479	0.243	32.31	0.6808	0.4093
Trt4 × Trt3	3	1	1.5404	1.3002	0.365	59.666	1.4037	0.2361
Trt4 × Trt3	4	1	-18.307	35348	<0.001	>999.9	0.0000	0.9996

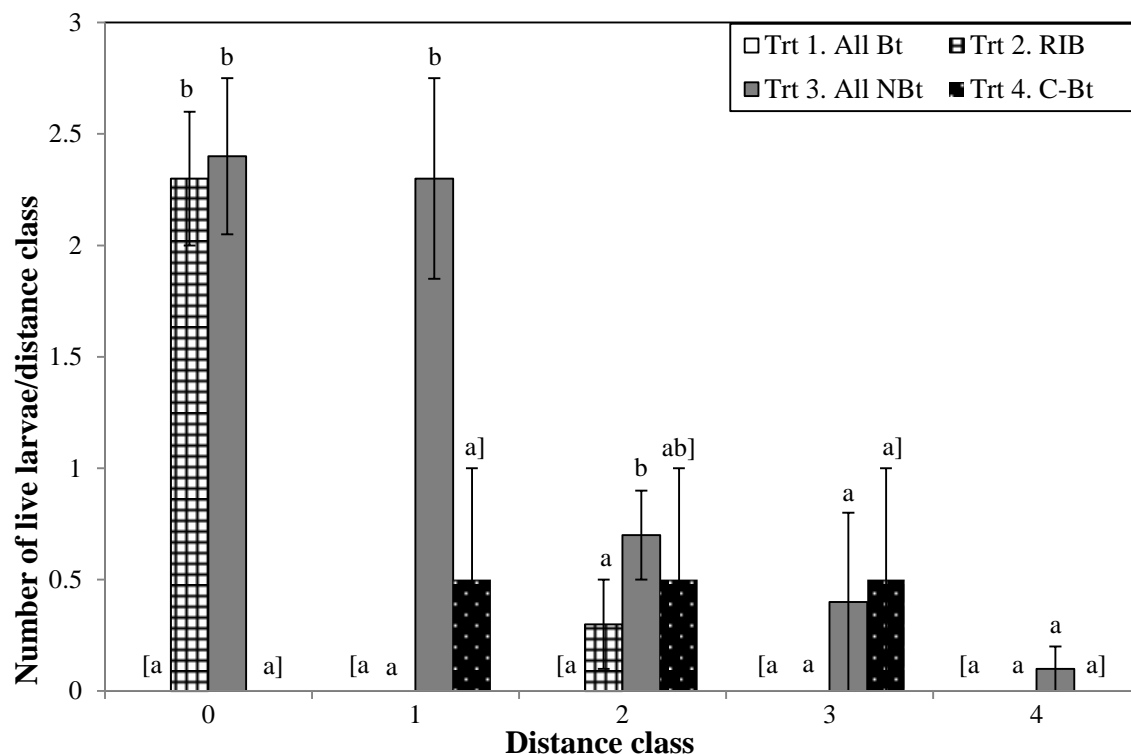
§ Trt 1= pure stand of 27 SmartStax™ plants (All Bt), Trt 2= one non-Bt plant in the center surrounded by 26 SmartStax™ plants (RIB), Trt3= pure stand of 27 non-Bt plants (All NBt), and Trt 4= one SmartStax™ plant in the center surrounded by 26 non-Bt plants (C-Bt).

- Indicates observations having non-positive frequencies or weights

The table shows distance classes (Class), parameter estimates (Estimates), and corresponding degrees of freedom (DF), Wald's 95% confidence limits (CL), chi-square value and the associated probability of obtaining a larger chi-square value (P> ChiSq).

### 3.3.2.2. Interplant and Inter-row Movement of *D. saccharalis* in Different Planting Patterns of Non-Bt and Bt Plants

As observed in the greenhouse study, because there were few survivors in the pure stand of Bt plants after 21-day of egg infestation, data used in ANOVA for inter-row movement of *D. saccharalis* did not include this planting pattern. Percentage of larvae that moved off the infested plants in the open field studies was also significantly different ( $F=5.68$ ;  $df = 2, 12$ ;  $P= 0.0184$ ) among the three planting patterns. However infested row abandonment was not significantly



**Fig. 3.5.** Occurrence of *Diatraea saccharalis* in four planting patterns of Bt and non-Bt corn (mean  $\pm$  sem). Distance class 0 refers to the center infested plants (focal plants); distance class 1 refers to all eight plants that are 1 plant away, distance class 2 refers to all six plants that are 2 plants away, distance class 3 refers to all six plants that are 3 plants away, and distance class 4 refers to all six plants that are 4 plants away from the central infested plant. Mean values followed by a same letter within the same distance class in brackets are not significantly different ( $P < 0.05$ ; LSD test).

different ( $F=1.71$ ;  $df = 2, 12$ ;  $P<0.2214$ ) for inter-row movement (Table 3.4)

The percentage of larvae that migrated off the central infested plants and survived on the surrounding plants in the open field study was very similar to that observed in the greenhouse study described above. There were no insects were recovered from the central infested Bt plants in Trt 4 compared to 93.0% of the survivors that did not move away from the central infested plants in “RIB”. Furthermore, a significantly lower ( $P < 0.05$ ) percentage (48.0%) of larvae moved from the center non-Bt plants and survived in the surrounded non- Bt plants in the structured refuge planting. However, there was no significant difference in the percentage



(0-33.4%) of larvae that moved away from the infested rows and survived on the adjacent rows among the three planting patterns.

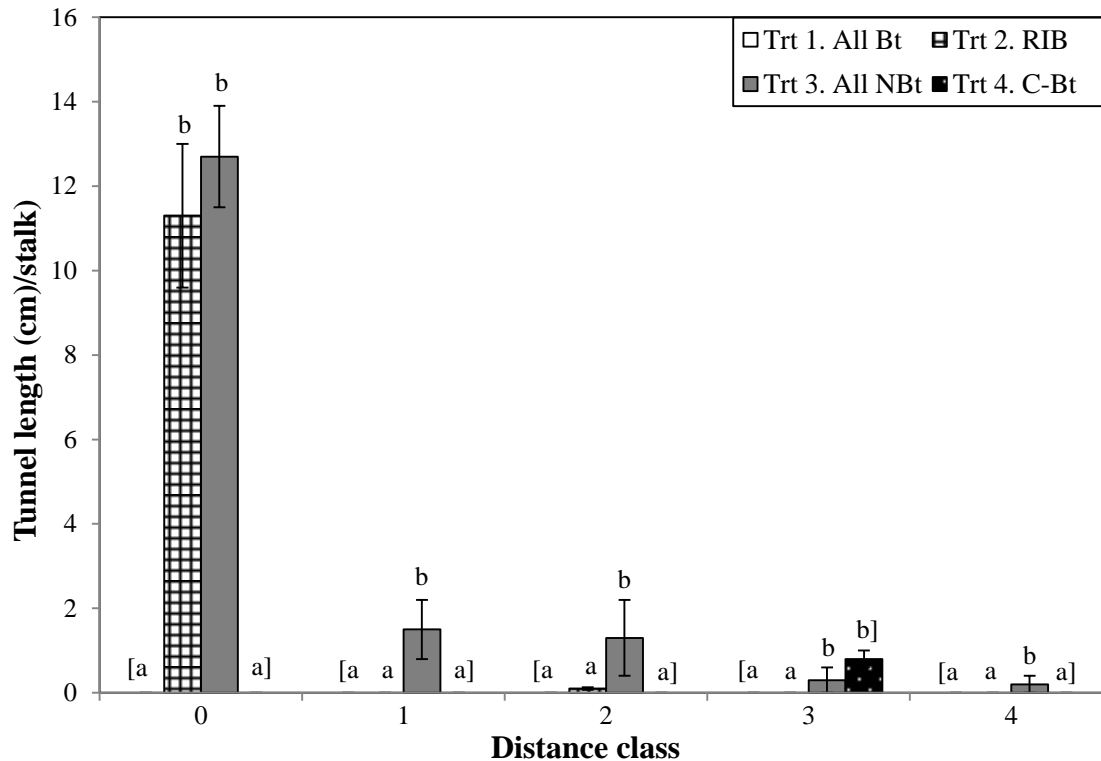
**Table 3.4.** Interplant and inter-row larval dispersal (mean $\pm$  sem) of *Diatraea saccharalis* in open field plants with artificial infestations of 50 ready-to hatch eggs on center plants in 2011

	Percentage dispersal*	
	Off the central infested plants	Dispersal to adjacent rows
Trt 1. All Bt plants	-	-
Trt 2. RIB	6.95 $\pm$ 4.5 a	0.0 $\pm$ 0.0 a
Trt 3. All Non Bt plants	48.0 $\pm$ 14.1 ab	8.6 $\pm$ 8.6 a
Trt 4. Center Bt	100.0 $\pm$ 0.0 b	33.4 $\pm$ 33.4 a
F	5.68	1.71
df	2, 12	2, 12
P- value	0.0184	0.2214

\* Means ( $\pm$  SE) followed with the same letter within a column are not statistically different ( $P < 0.05$ ; LSD test)

### 3.3.2.3. Plant Injury of *D. saccharalis* in Different Planting Patterns of Non-Bt and Bt Plants

Tunnel length was highly correlated with the number of live insects recovered at each distance class in the four planting patterns. Tunnel length in plants at 0<sup>th</sup> and 1<sup>st</sup> distance classes were significantly different among planting patterns ( $F \geq 3.79$ ;  $df = 3, 17$ ;  $P \leq 0.0299$ ) but not significant at the greater distances ( $F \leq 2.18$ ;  $df = 3, 17$ ;  $P \geq 0.1277$ ). Inside the stalks of the central plants, an average tunnel length of 12.7 cm/plant was observed in the pure stand of non-Bt plants which was not significantly different from that (11.3 cm) of the central plants in “RIB” planting, while no tunnels were found in the other two planting patterns. At the distance classes 1 and 2, an average tunnel length of 1.3 -1.5 cm/plant was recorded in the pure stand of non-Bt plants which was significantly greater than that (0- 0.1 cm) of the other three planting patterns (Fig. 3.6).



**Fig.3.6.** Tunneling length (cm/stalk, mean $\pm$  sem) caused by *Diatraea saccharalis* in four planting patterns of Bt and non-Bt corn after 21 days infested with 50 ready-to-hatch eggs. Comparisons were made within a distance class among planting patterns. Distance class 0 refers to the center infested plants (focal plants); distance class 1 refers to all eight plants that are 1 plant away, distance class 2 refers to all six plants that are 2 plants away, distance class 3 refers to all six plants that are 3 plants away, and distance class 4 refers to all six plants that are 4 plants away from the central infested plant. Mean values followed by a same letter within the same distance class are not significantly different ( $P < 0.05$ ; LSD tests).

### 3.3.3. Open Field Tests with Artificial Infestation of Neonates of *D. saccharalis* on All Plants

#### 3.3.3.1. Occurrence of *D. saccharalis* in Different Planting Patterns of Non-Bt and Bt Plants

The number of insects that survived after 21 days of artificial infestation of 10 neonates/plant was significantly different among the treatments ( $F=2.53$ ;  $df= 5, 24$ ;  $P= 0.0367$ ). Genuity<sup>®</sup> SmartStax<sup>™</sup> plants were excellent for controlling *D. saccharalis*. No live insects were found in the pure stand of Bt plants and only 0.02 insects/plant were recorded in the Bt plants of

the “RIB” planting. An average of 0.86 live insects/plant was found in the pure stand of non-Bt plants which was similar to that of the central Bt plants (0.6 insects/plant) or the non-Bt plants (0.68 insects/plant) in Trt 4 or that (0.4 insects/plant) of the central non-Bt plants in the “RIB” planting (Table 3.5).

### 3.3.3.2. Plant Injury of *D. saccharalis* in Different Planting Patterns of Non-Bt and Bt Plants

The length of tunnels in stalks of corn plants in the open field study with artificial infestation was not highly correlated to the larval occurrence after 21 days of release of neonates. Stalk tunnel length was significantly different among treatments ( $F=1.43$ ;  $df=5, 24$ ;  $P=0.0248$ ). Tunnel length (8.2 cm/plant) in center non-Bt plants in the “RIB” planting was significantly greater than that of any other plants including the non-Bt plants in the pure stand of non-Bt plants (Table 3.5). An average of 1.54 cm tunnel was observed per plant in pure stand of non-Bt plants which was not significantly different (0.9 cm) to that of non-Bt plants in Trt 4. No tunnels were found in pure stand of Bt plants and few tunnels (0.06-0.4 cm) were in any other Bt plants.

**Table 3.5.** Larval occurrence and stalk tunnel length of *Diatraea saccharalis* in different planting patterns in open field tests with artificial infestation of 10 neonates /plant

Planting pattern		No. Larvae/ plant*	Tunnel length (cm)/ plant*
Trt 1. Pure stand of Bt plants		$0.0 \pm 0.0$ a	$0.0 \pm 0.0$ a
Trt 2. One non-Bt plant in the center surrounded by 26 Bt plants (RIB)	Bt plants	$0.02 \pm 0.02$ a	$0.06 \pm 0.06$ a
	Non- Bt plant	$0.4 \pm 0.13$ ab	$8.2 \pm 1.0$ c
Trt 3. Pure stand of non-Bt plants (structured refuge)		$0.86 \pm 0.15$ b	$1.54 \pm 0.5$ b
Trt 4. One Bt plant in the center surrounded by 26 non-Bt plants	Bt plant	$0.6 \pm 0.1$ ab	$0.4 \pm 0.1$ a
	Non- Bt plant	$0.68 \pm 0.12$ b	$0.9 \pm 0.4$ b

\* Means followed by the same letter are not significantly different (LSD,  $P > 0.05$ ).

### 3.4. Discussion

In pure stand of Bt plants, there were no survivors of *D. saccharalis* and no tunnels inside the stalks after 21 days of egg/larval infestations in all three tests of this study. Similarly, in the “RIB” planting, few insects survived and caused just very little injury on Bt plants in the three tests. The results showed that the transgenic plants containing Genuity® SmartStax™ traits was excellent for controlling *D. saccharalis* on corn and protecting the plant from insect damage. The results of the current study were consistent with the results observed in a previous greenhouse study with artificial infestation of three genotypes of *D. saccharalis*, which showed that Genuity® SmartStax™ Bt corn hybrids were very effective against all the three genotypes including a Cry1Ab-susceptible strain, a Cry1Ab-resistant strain, and a heterozygous genotype (Chapter 2).

Larval movement of corn stalk borers in corn field appears to be very common. Studies on *O. nubilalis* have shown that 50- 56% of the neonates during the first 48 hours after hatching abandoned the primary host plants and dispersed to other plants along the infested row as well as to plants in adjacent rows (Ross and Ostlie, 1990). After this period, approximately 85-94% remained within the infested rows when sampling was done 21 days after infestation (Ross and Ostlie, 1990). For this reason, larval dispersal of *D. saccharalis* in this study was examined by infesting eggs on plants to simulate natural conditions. Results of the current study indicated that the dispersal rate of *D. saccharalis* in pure stand of non-Bt corn plants could vary in different test conditions. It ranged from 48% off from infested plants and survived in surrounded plants in the open field tests to 92% in the greenhouse conditions. The results were a little surprising because wind should be stronger in the open field conditions than in the greenhouse conditions, which

should create a more favorable condition for larval dispersal in the open field than in the greenhouse. Nevertheless, both greenhouse and open field tests showed that larvae of *D. saccharalis* have the ability to move from infested plants to at least 4-plants away with a majority of larvae staying within 3-plants away from infested plant. Larvae of *D. saccharalis* can move from the infested rows to the adjacent rows although the intensity of dispersal also varied depending on the test conditions.

The current study also indicated that larval dispersal behavior of *D. saccharalis* could be different in different planting patterns of non-Bt and Bt corn plants. Previous studies showed that larval dispersal of *O. nubilalis* has been seen as silking or walking. As in the case of silking, neonates of *O. nubilalis* secrete silks which they use to hang from the host plant tissue to reach other tissues of same host or come in contact with other plant tissues (Bell et al., 2005). In some cases, the silk is laid in strands hanging down the host plant but well inclined to the air currents that drag the neonates to the adjacent host plant (Zalucki et al., 2002; Bell et al., 2005; Goldstein et al., 2010). Neonates of *O. nubilalis* can employ several pre- dispersal behavioral responses ahead of making a suitable host plant to feed on. As other lepidopteran larvae, they move about during leaf exploration phase to most conducive surfaces/plant tissues in the leaf whorl or leaf tissues and feed on preferred tissues. The chances of the larvae migrating from the focal plant to new adjacent plants only to find them not suitable (e.g. Bt plant tissue) then move back to the ancestral host plant are highly predictable. The ability of the neonates of *O. nubilalis* to assess the host quality leading to either acceptance or un-acceptance is the primary means for feeding and silking on suitable host plants (e.g. Bt plants) (Goldstein et al., 2010). Such food selection behaviors could result in different dispersal behavior of *D. saccharalis* in different planting patterns of Bt and non-Bt corn.

In spite of different dispersal behaviors in different planting patterns of Bt and non-Bt corn, there were no significant differences ( $P>0.05$ ) in number of larvae of *D. saccharalis* recovered from the central infested plants between the pure stand of non-Bt corn (structured refuge) and the “RIB” planting in both the greenhouse and open field tests. Plant injury (tunnel length inside the stalks) was also similar in the central infested plants between the two planting patterns in both tests. In the open field study with artificial infestation of neonates on all plants, the number of *D. saccharalis* recovered from the central non-Bt plants in the “RIB” planting was low by approximately 50% compared to that observed in the pure stand of non-Bt plants (structural refuge) but the difference was not significant ( $P>0.05$ ). In addition, occurrence and larval movement of *D. saccharalis* in different planting patterns have been evaluated in two previous tests. One test was conducted in an open field condition with natural infestation of *D. saccharalis* in 2009 and another was carried out in the greenhouse in 2010 with the same experimental design and procedures as described in section 3.2.3 of the current study. The results of both the previous tests showed that the number of *D. saccharalis* individuals found in the non-Bt refuge plants in the “RIB” planting was not significantly different compared to the insect populations on the plants of “structured refuge” planting (BRL, FH, unpublished data). Several early studies discussed seed mixture strategy. Gould and Anderson (1991) suggested that seed mixture strategy could be successful in delaying the development of insect resistance against Bt crops. Furthermore, seed mixture was predicted to have ability of enhancing random mating between insects within the field if larval movement among Bt and not-Bt plants was not a significant event (Showers et al., 1976). Mallet and Porter (1992) reported that if insect movement was independent of presence of toxin inside plants, Bt and non-Bt seed mixtures could be used to delay resistance development for Bt crops. Together with other data, the results

indicate that the refuge plants in the seed mixture strategy might be able to provide a similar population of susceptible *D. saccharalis* as the “structured refuge” design.

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## CHAPTER 4. SUMMARY AND CONCLUSIONS

The sugarcane borer, *Diatraea saccharalis* (F.), is an important pest of field corn in the U.S. mid-southern region, especially in Louisiana and the Gulf Coast area of Texas. Like other areas in the U.S., planting of transgenic *Bacillus thuringiensis* (Bt) corn is currently the primary tool for managing corn stalk borers including *D. saccharalis* in the mid-southern region. The foremost single- gene Bt corn was introduced in the U.S. mid southern regions in 1999 for management of corn stalk borers. One of the major threats to the sustainable use of Bt crops is resistance development in target insect pest populations. Resistance can develop rapidly if there is a high selection pressure, failure to comply with refuge requirements, and use of non- high dose products. The rapid adoption of Bt corn hybrids and the increased problems of *D. saccharalis* in the mid-southern region demands an effective insecticide resistance management (IRM) plan for the sustainable use of the Bt corn technologies in this region. During 2010-2011 crop seasons, transgenic corn technologies (e.g. Genuity® SmartStax™, Agrisure® Viptera™ 3111) expressing multiple dissimilar Bt proteins that target lepidopteran pests were first commercially planted in the United States. The use of pyramided Bt corn hybrids is expected to delay resistance development in target insect populations. Because of the compliance issue in the use of the “structured refuge” for resistance management, the U.S. EPA has approved a seed mixture refuge strategy (also called “refuge-in-the-bag” or RIB) for planting pyramided Bt corn hybrids in the north U.S. Corn Belt where no cotton is planted. The “RIB” strategy has not been approved in the south region where cotton is also planted. Information to support the use of the pyramided Bt corn technologies along with “RIB” refuge strategy for managing *D. saccharalis* is limited. The objectives of this study were 1) to evaluate larval survival of Cry1Ab-susceptible (Cry1Ab-SS), -resistant (Cry1Ab-RR), and -heterozygous (Cry1Ab-RS) genotypes of *D.*

*saccharalis* on transgenic corn containing single or pyramided Bt genes to determine if the pyramided Bt corn could overcome the Cry1Ab resistance in *D. saccharalis* and 2) to investigate larval movement of *D. saccharalis* in different planting patterns of non-Bt and Bt corn to determine if “RIB” is an appropriate approach for providing refuge for managing *D. saccharalis* with pyramided Bt genes.

In this study, performance of Cry1Ab-SS, -RS, and -RR genotypes of *D. saccharalis*, on five commercial corn hybrids were evaluated with leaf tissue bioassays in the laboratory and intact plants in the greenhouse during 2010-2011. The five hybrids included two non-Bt and three Bt corn hybrids representing three transgenic technologies, YieldGard<sup>®</sup>, Genuity<sup>®</sup> VT Triple Pro<sup>™</sup> and Genuity<sup>®</sup> SmartStax<sup>™</sup>. YieldGard<sup>®</sup> corn expressed a single Bt protein (Cry1Ab), while Genuity<sup>®</sup> VT Triple Pro<sup>™</sup> contained Cry1A.105 and Cry2Ab2 for controlling above-ground lepidopteran pests and Cry3Bb1 for managing below-ground rootworms. Genuity<sup>®</sup> SmartStax<sup>™</sup> produced all the three Cry proteins of Genuity<sup>®</sup> VT Triple Pro<sup>™</sup> as well as Cry1F targeting above-ground lepidopteran pests and Cry34/35Ab1 against below-ground rootworms.

Leaf tissue bioassays in the laboratory showed that 6-day larval survival of *D. saccharalis* on non-Bt leaf tissue was not significantly different between the two non-Bt corn hybrids and across the three insect genotypes with an average survivorship of 75.5%. Only a very low survivorship (3%) of Cry1Ab-SS larvae was observed on leaf tissue of YieldGard<sup>®</sup> plants. Larvae of Cry1Ab-RR on YieldGard<sup>®</sup> corn leaf tissue demonstrated a 32% survivorship after 6 days, which was significantly greater than that of the Cry1Ab-SS. An average of 19% larvae of Cry1Ab-RS genotype also survived after 6 days on YieldGard<sup>®</sup> corn leaf tissue. Leaf tissue of both pyramided Bt corn hybrids were excellent against *D. saccharalis*. All larvae were killed after 6 days on leaf tissue removed from the two pyramided Bt corn hybrids.

Two independent trials were conducted to evaluate the performance of the three insect genotypes of *D. saccharalis* on intact plants in the greenhouse. In the tests, larval survival, entry/exit holes on stalks, and tunnel length inside stalks were recorded 21 days after infestation of 20 (first trial) or 10 (2<sup>nd</sup> trial) neonates of *D. saccharalis* on each potted plant. After 21 days, 42.6-62.5% of larvae survived on non-Bt corn plants. Larval survivorship rates on YieldGard<sup>®</sup> plants were 4.7-5.6% for Cry1Ab-SS, 29.4-32.5 % for Cry1Ab-RS, and 36.6-45.6% for Cry1Ab-RR. Both pyramided Bt corn hybrids were very effective against *D. saccharalis* regardless of the insect genotypes. The 21-day survivorship rate on the two pyramided Bt corn hybrids was <2% for Cry1Ab-SS and Cry1Ab-RS, and <5% for Cry1Ab-RR. Larvae of Cry1Ab-RS and -RR caused significant entry/exit holes and tunneling inside the plant stalks of non-Bt and YieldGard<sup>®</sup> corn plants, while they just produced little injury on the two pyramided Bt corn hybrids. The results generated from the leaf tissue bioassays in the laboratory and intact plant tests in the greenhouse showed that the Cry1Ab-resistant *D. saccharalis* was highly resistant to YieldGard<sup>®</sup> corn and the resistance to YieldGard<sup>®</sup> corn was functionally incompletely dominant.

Larval movement, occurrence, and plant injury of *D. saccharalis* were evaluated in four planting patterns of Bt and non-Bt plants in greenhouse and field conditions. Each planting pattern consisted of 3 rows and 9 plants in each row (a total of 27 plants). The four different planting patterns were: Trt 1) pure stand of 27 SmartStax<sup>™</sup> plants, Trt 2) one non-Bt plant in the center surrounded by 26 SmartStax<sup>™</sup> plants, Trt 3) pure stand of 27 SmartStax<sup>™</sup> plants, and Trt 4) one SmartStax<sup>™</sup> plant in the center surrounded by 26 non-Bt plants. The planting pattern of Trt 2 was designed to simulate a 96:4% “RIB”, which was close to the currently used “95:5%” “RIB” for planting Genuity<sup>®</sup> SmartStax<sup>™</sup> corn in the United States, while Trt 3 was used to simulate a “structured refuge” planting. Studies were conducted in three conditions: 1)

greenhouse with artificial infestations of 50 eggs on the center plants, 2) open field with artificial infestations of 50 eggs on the center plants, and 3) open field study with artificial infestations of 10 neonates on every plant. Larvae of *D. saccharalis* showed the ability to move from infested plants to at least 4-plants away and from the infested rows to adjacent rows. In each tests, number of live insects and stalk tunnel length in each plant were checked after 21 days of insect infestation.

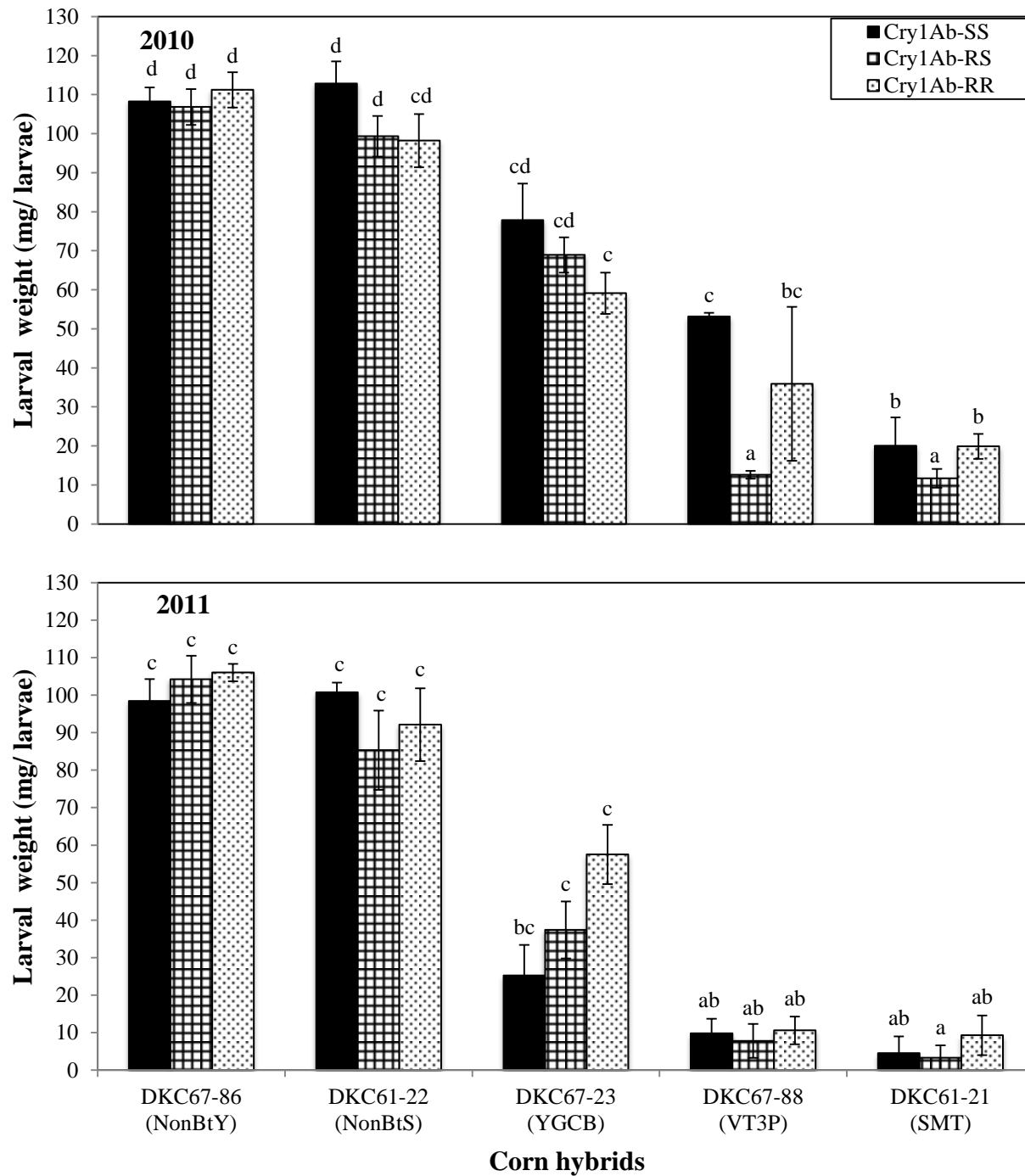
Both tests with artificial infestation of eggs on the central plants showed that the dispersal rate of *D. saccharalis* in pure stand of non-Bt corn plants could vary in different test conditions, dispersal ranged from 48% (off the infested plants and survived in surrounding plants) in the open field tests to 92% in the greenhouse conditions. Both tests also demonstrated that larvae of *D. saccharalis* have the ability to move and survive from infested plants to at least 4-plants away with a majority of larvae staying within 3-plant distance. Larvae of *D. saccharalis* can move and survive from the infested rows to the adjacent rows although the intensity of dispersal also varied depending on the test conditions. Larval dispersal behavior of *D. saccharalis* could also be different in different planting patterns of non-Bt and Bt corn plants. There were no significant difference ( $P>0.05$ ) in number of larvae of *D. saccharalis* recovered from the central infested plants between the pure stand of non-Bt corn (structured refuge) and the center non-Bt plants in “RIB” planting in both tests. Plant injury (tunnel length inside the stalks) was also similar in the central infested plants between the two planting patterns in both tests. In the open field study with artificial infestation of neonates on all plants, the number of *D. saccharalis* recovered from the central non-Bt plants in the “RIB” planting was approximately 50% of population found in the pure stand of non-Bt plants (structured refuge). Occurrence and larval movement of *D. saccharalis* in different planting patterns have been evaluated in two previous tests.

One test was conducted in an open field condition with natural infestation of *D. saccharalis* in 2009 and another was carried out in the greenhouse in 2010 with the same experimental design and procedures as described in section 3.2.3 of the current study. The results of both the previous tests showed that the number of *D. saccharalis* individuals found in the non-Bt refuge plants in the “RIB” planting was not significantly different compared to the insect populations on the plants of the “structured refuge” planting.

In summary, the results of this study showed that corn hybrids containing Genuity® VT Triple Pro™ or Genuity® SmartStax™ traits were very effective for controlling *D. saccharalis*. The highly resistant strain of *D. saccharalis* on YieldGard® corn- was also susceptible to the two pyramided Bt corn hybrids, suggesting that the pyramided Bt corn can overcome the Cry1Ab resistance and thus should offer as a means for Cry1Ab resistance management in *D. saccharalis*. The results of this study also indicate that the seed mixture strategy might be able to provide a similar population of susceptible *D. saccharalis* as the “structured refuge” design. Results of current study, together with the previous data, support the use of pyramided Bt corn for managing *D. saccharalis* in the mid-southern region of the United States.

## APPENDIX: SUPPLEMENTAL DATA

Weight of *Diatraea saccharalis* larvae recovered on intact greenhouse plants in 2010 and 2011 study after 21 days of infestation





Body weight (mg/ larvae, mean  $\pm$  sem) of larvae of Cry1Ab-susceptible (Cry1Ab-SS), -heterozygous (Cry1Ab-RS), and -resistant (Cry1Ab-RR) genotypes of *Diatraea saccharalis* recovered after 21 days of infestation on two non-Bt corn and three Bt corn hybrids containing single or multiple Cry proteins during 2010 and 2011 trials. Mean values followed by the same letter are not significantly different ( $P < 0.05$ ; LSMEANS test).

## **VITA**

David Sindani Wangila is the 8<sup>th</sup> child of Mr. Dismas Wangila and Mrs. Melap Naliaka. He was born and raised in Malakisi, Bungoma County in Kenya in 1984. He attended Teremi High school and graduated in 2003. He was awarded a tutorial assignment in a nearby village-secondary school for one year before being admitted to Masinde Muliro University of Science & Technology in 2005 to pursue undergraduate studies in Education Science. He received a Bachelor of Education Science degree in 2009 majoring in biology and minor in chemistry.

He was hired on the eve of his last undergraduate final examinations as Biology/Chemistry teacher at Moding' High school and he held this teaching assignment for one year (2009-2010) before being awarded a research assistantship for a Master's degree in Entomology at Louisiana State University.

In May 2010, Mr. Wangila began his master's studies in the Department of Entomology at Louisiana State University. His thesis research with Dr. Fangneng Huang has focused on the evaluation of Bt corn expressing pyramided traits for management of sugarcane borer, a devastating pest of field corn in the U.S. mid-southern region. He is currently completing the requirements for the degree of Masters of Science which will be conferred on the May 18<sup>th</sup>, 2012 commencement ceremony. Mr. Wangila currently has an admission to a doctoral program in Entomology at University of Nebraska- Lincoln starting in June, 2012.