BEHAVIORAL CHARACTERISTICS OF NEONATE EUROPEAN CORN

BORER, *OSTRINIA NUBILALIS*, ON BT CORN

by

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fulfillment of the requirements for the degree of Master of Science in Entomology

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ABSTRACT

European corn borer (ECB), *Ostrinia nubilalis* (Hübner)*,* is an economically important insect pest of corn in the US and Canada. The development of genetically modified corn expressing genes derived from *Bacillus thuringiensis* (Bt) that encodes insecticidal crystalline (Cry) proteins has proven to be effective in controlling this insect. The purpose of this study was to assess dispersal and feeding behavior of neonate ECB on Bt corn. I examined differences in neonate ECB dispersal behavior for the first four hours following eclosion in the field among stacked pyramid (DAS 59122-7 × TC1507 × MON810) Bt corn, non-Bt near isoline, and non-Bt sweet corn; and in the laboratory among Bt corn hybrids TC1507, MON810, a pyramid combining TC1507 and MON810, and non-Bt near isoline corn. I also examined differences in feeding behavior between MON810 Bt corn and non-Bt near isoline corn for four intervals over a 48-hour period. In field experiments, I found that dispersal was significantly higher on non-Bt near isoline and Bt corn when compared to sweet corn. In laboratory experiments, dispersal was significantly higher on MON810 and stacked pyramid Bt corn than on non-Bt near isoline corn. Finally, feeding experiments suggested that there was a significant difference in feeding behavior between MON810 Bt corn and non-Bt near isoline corn. The findings from the feeding experiment also suggested that more than 50% of the larvae abandoned the plant before there was evidence of feeding for both Bt and non-Bt corn. Results indicated that a mixed refuge method could prove to be an effective solution for managing ECB resistance on Bt corn. However, additional research needs to be conducted on ECB larval movement between Bt and non-Bt corn to fully assess the efficacy of the mixed refuge strategy.

INTRODUCTION

Life History and Ecology

The European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is an

economically important insect pest of corn and other crops in the United States and

Canada. The ECB is an introduced insect species from Europe, and is thought to have

arrived in North America during the early 1900s in broom corn imported from

Hungary and Italy for the manufacture of brooms (Mason et al. 1996). The ECB was

first reported in North America in 1917 in Massachusetts, and also was later found in

1921 near Lake Erie. Since its initial discovery, the insect has spread north into

Canada, south to Florida, and westward across the United States to the Rocky

Mountains (Mason et al. 1996).

ECB is a generalist herbivore and has been reported to infest more than 200

species of plants (Hodgson 1928), including corn, cotton, wheat, peppers, beans,

tomatoes, potatoes, and many other herbaceous plants, including some weeds (Dwyer

et al. 2001). Although the species is capable of surviving in herbaceous plants with

stems large enough for larvae to enter, the ECB has a strong preference for corn as a

host plant (Pedigo and Rice 2009).

In the US, ECB can have as many as 4 generations per year, depending on the

region (Velasco et al. 2002). In most of the major corn growing areas of North

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America, ECB has two generations per year. The first seasonal generation of ECB

begins with full-grown larvae, which hibernated through the winter in corn stalks and

cobs. In Delaware, the over-wintering larvae pupate in mid to late April. Adults

emerge in late May through June and mate in grassy areas near the cornfield (Pedigo

and Rice 2009). Egg masses are deposited on the underside of leaves, and generally

contain 15-30 eggs per egg mass. Larvae emerge from the egg mass in three to seven

days depending on temperature. The first generation of ECB infests young plants and

feeds on whorl leaves, causing pinhole-sized damage (Velasco et al. 2002). Larvae

move to the stalk and bore into the plant once the third instar is reached (Edwards et

al. 1998). ECB larvae normally go through five stadia before pupating in the stalk of a

host plant. First-generation larvae pupate in July and produce adults that lay eggs for

the second generation. Second generation larvae infest older corn plants, damaging

stems and ears (Velasco et al. 2002). Most yield losses can be attributed to

physiological damage caused by larval feeding in leaf and conductive tissues, which

can impair the ability of plants to produce normal amounts of grain (Mason et al.

1996).

Management Strategies

Damage from ECB infestations on corn has been estimated to exceed $1 billion

annually in yield losses and control costs (Mason et al. 1996). In most US corn

growing regions, it is estimated that a single ECB larva can cause between 3% and 7% yield loss per plant (Koziel et al. 1996). Traditionally, control has relied on the introduction of biological control agents, multiple insecticide applications, and corn hybrids bred for intermediate resistance to ECB (Reed and Halliday 2001). Several biocontrol agents have been introduced from Europe to the United States. However, very few have become established and rearing procedures can be expensive (Mason et al. 1996).

Chemical insecticides can be used to control ECB damage on corn and, if timed well, typically provide control for 60 to 95% of first generation larvae and 40 to 80% of second generation larvae (Ostlie et al. 1997). However, insecticides are more effective when the larvae have just hatched or when they migrate to neighboring plants. Once the larvae bore into the ears or stalk of the plants, insecticides offer little control. Additional problems with insecticide use are that it is expensive and raises health, safety, and environmental concerns. Also, scouting in the field multiple times each summer to locate egg masses and larvae for determining if insecticidal applications are needed can take time and requires skill (Velasco et al. 2002).

The plant alkaloid, 2-4 dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), was found to be a natural feeding deterrent and has been incorporated in many corn hybrids as an antibiosis agent for resistance to ECB. Although host plant

resistance to first-generation ECB has been satisfactory, a major limitation of using DIMBOA for resistance has been the lack of protection from second-generation attack (Reed and Halliday 2001). The concentration of DIMBOA in a corn hybrid usually decreases as the plant develops, and therefore, provides minimal resistance to second-generation ECB which damage older plants (Mason et al. 1996).

Transgenic corn hybrids containing specific insecticidal proteins have become an effective method of controlling the ECB. *Bacillus thuringiensis* (Bt) is a naturally-occurring soil bacterium that produces crystal-like proteins. There are 13 insecticidal proteins of Bt strains that kill different groups of insects (Pedigo and Rice 2009). Bt in insecticidal formulations is the most widely used biological control agent in North America. The Bt subspecies *kurstaki*, which has crystal proteins that are effective against Lepidoptera, have been used by organic growers for many years. The crystalline (Cry) proteins are insect stomach poisons that must be eaten to kill the insect. Once eaten, an insect's own digestive enzymes activate the toxic form of the protein. The Cry proteins bind to specific "receptors" on the intestinal lining and rupture the cells, causing the insect to stop feeding and eventually die (Schnepf et al. 1998). These proteins have no effect on vertebrates because there are no binding sites specific for the Cry proteins from *B. thuringiensis* on the surface of vertebrate intestinal cells (Koul and Dhaliwal 2004).

Bt has several subspecies and among these scientists have cultured a variety of strains that express different proteins. For example, the subspecies *kurstaki* makes Cry1Ab, Cry1Ac, and Cry1C which are toxic to the larvae of certain species of moths and butterflies, including ECB (Huang et al. 2002). Cry1F produced by Bt subspecies *aizawai* is expressed in Event TC1507 corn and also protects corn against certain lepidopteran insect larvae including ECB (EPA 2005a). There are other Bt proteins that provide control for coleopteran pests, including Cry34Ab1 and Cry35Ab1which are expressed in Event DAS-59122-7 corn to offer protection against corn rootworm (EPA 2005b). These proteins are often very specific to target insect species (Huang et al. 2002).

In 1996, Bt corn hybrids were first planted commercially in the Midwestern United States (Pierce et al. 2001), and by 2009, various Bt corn hybrids were planted on 22.3 million hectares, representing 63% of the United States corn acreage (USDA-NASS 2009). Koziel et al. (1993) reported the first field results for event 176 Bt corn, in which effective control of the ECB was achieved without pesticide applications. Untreated non-Bt corn was damaged with severe leaf feeding and stalk tunneling, whereas those expressing Bt had very little damage. It has been estimated that approximately 99% control of ECB can be obtained when planting Bt corn that has Cry proteins targeted for this insect (Baute et al. 2002). In terms of economic returns,

Stanger and Lauer (2006) reported that Bt corn yielded 7% greater and had 22% less

lodging than non-Bt corn, but the yield and lodging benefits of Bt hybrids may be

offset by the higher seed and harvest costs. Overall, Bt corn produces Cry proteins at

highly effective dose throughout the plant and provides consistent season-long control

of ECB and other lepidopteran pests at about one-fifth the cost of an insecticide

application (Dillehay et al. 2005).

The high efficacy of Bt corn has resulted in rapid and widespread adoption

(Hurley et al. 2002). The potential for development of resistance in ECB populations

poses a problem for effective control of larval damage on corn crops. Although field

populations of ECB have not yet shown resistance to Bt corn, resistance to Bt toxins in

ECB has been demonstrated in laboratory-selected strains of this insect exposed to the

Bt formulation Dipel and to the single toxins Cry1Ac and Cry1Ab (Alves et al. 2006).

Recently, strong evidence of field-evolved resistance to the Bt toxins in transgenic

crops has been reported for some populations of three noctuid moths: maize stalk

borer, *Busseola fusca*; corn earworm, *Helicoverpa zea*; and fall armyworm,

Spodoptera frugiperda (Tabashnik et al. 2009). Field-evolved resistance of *S.*

frugiperda to Bt corn producing Cry1F occurred in four years in Puerto Rico making

this the fastest documented case of field-evolved resistance to a Bt crop (Tabashnik et

al. 2009). Also, resistance to the Bt toxins in foliar sprays has evolved in field populations of diamondback moth, *Plutella xylostella* (Alves et al. 2006).

Resistance management is recognized as essential to the long-term effectiveness of Bt crops for ECB control. Resistance management in Bt corn for ECB is currently based on two complementary principles: high dose and refuge. The intent of designing Bt corn to produce highly effective dose of Bt Cry proteins is to kill all ECB larvae with no genes for resistance (ss) as well as larvae with one copy of a resistance gene (rs). In general, expression of highly effective dose of Cry proteins in Bt crops is not required in all resistance management practices. However, the high dose strategy has been effective in delaying the development of resistance in ECB populations when implemented in concert with a refuge. The purpose of the refuge population is to provide a source of susceptible (ss) ECB not exposed to Bt corn that could mate with potentially resistant (rr) moths emerging from nearby Bt corn in order to reduce the frequency of resistant genes (Ostlie et al. 1997).

Refuges should not only increase the possibility of genetic exchange among resistant and susceptible ECB but also allow for the persistence of natural enemies (Pierce et al. 2001). All immature stages of the ECB are attacked by a number of naturally occurring predators and parasitoids (Mason et al. 1996). These natural enemies, along with abiotic factors, contribute to a relatively high natural mortality

(60-90%) of ECB eggs and early instars (Kuhar et al. 2002). Predators of ECB include

predaceous mites (Acari: Phytoseiidae), flower bugs and minute pirate bugs

(Hemiptera: Anthocoridae), green lacewings (Neuroptera: Chrysopidae), ground

beetles (Coleoptera: Carabidae), and lady beetles (Coleoptera: Cocinellidae). The

parasitoids include *Lydella thompsoni* (Diptera: Tachinidae), *Eriborus terebrans*

(Hymenoptera: Ichneumonidae), and *Macrocentrus cingulum* (Hymenoptera:

Braconidae) (Bhalgat et al. 2004).

Another valuable option for resistance management, in combination with the

use of a refuge, is the expression of multiple Cry proteins targeted for a pest in crops

that provide different modes of action with respect to the insect's mechanism of

resistance. Cry toxins that recognize different receptors in the same target species

could be useful in delaying resistance in a population, since the pest is less likely to

develop cross-resistance (Schnepf et al. 1998). The combining of multiple transgenic

traits in the same crop variety has been termed as stacking or pyramiding (Koul and

Dhaliwal 2004). The concept of a "pyramid" specifically applies to Bt plants

producing two or more toxins with different modes of action (Bravo and Soberón

2008) targeted for a particular species or a group of closely related pests (Pedigo and

Rice 2009). Recently, transgenic corn expressing six Cry genes has been

commercially released. This Bt pyramid, named "SmartStax", contains three Cry

genes encoding toxins that target coleopteran pests and three Cry genes that encode

toxins against lepidopteran pests, in addition to two traits conferring herbicide

tolerance (Gatehouse 2008).

To ensure an adequate ratio of susceptible to resistant individuals, the US

Environmental Protection Agency has mandated that each field planted with Bt corn

be within one ½ mile from a refuge area of non-Bt corn that is equal to 20% of the

total area of the corn planted (EPA 1998, Losey et al. 2002). In November 2009 EPA

approved the first pyramid Bt product with refuge size of 5% of the acreage in the corn

belt and 20% in areas where corn is grown with cotton (EPA 2009). Refuge

deployment can occur under one of several recommended strategies: growing non-Bt

corn in an adjacent field no more than ½ mile away from the Bt corn, growing non-Bt

corn as a block within a Bt corn field, growing Bt and non-Bt corn as alternating strips

(a minimum of 4 rows) through a field, and growing the non-Bt corn refuge as field

edges around an entire field of Bt corn (Pilcher et al. 2001). Currently, refuges are

required to be in some kind of structured configuration (block or strip plantings) on the

farm. However, using this strategy can be inconvenient for growers and incurs a cost

to the producer in time, labor, and seed inputs, thus leading to concern about grower

non-compliance (Bates et al. 2005).

One concept being considered on a large scale is the implementation of an unstructured refuge that employs a mixture of Bt and untreated non-Bt corn seed throughout a field, commonly referred to as "refuge in a bag." In the field, plants that grow from the non-Bt corn seeds in the mix serve as a refuge for some of the corn borers. This produces a refuge population and ensures there will be corn borers not exposed to Bt available to mate with any Bt-resistant survivors. The offspring will likely not be resistant, which will slow the evolution of corn borers resistant to Bt (Comis 2003). A concern about this approach is that larvae which begin feeding on non-Bt plants might disperse to and feed on Bt plants, survive to adulthood, and produce offspring with partial tolerance for Bt toxins (Gould 2000). Huang et al. (1999) conducted tests on ECB survivorship on Bt corn and found that later instars of ECB are much more tolerant to *B. thuringiensis* than are early instars. Furthermore, both Walker et al. (2000) and Huang et al. (2002) found that some late-instar ECB could survive on Bt corn. Obviously, larvae that possess even a partial tolerance to Bt would survive better and would selectively contribute to producing offspring that are more tolerant. Over multiple generations, this would lead to stronger tolerance and eventual full resistance.

Dispersal and Feeding Behavior on Bt Corn

Most neonate lepidopterans have a prefeeding movement phase, described as either local leaf exploration or longer distance dispersal (Zalucki et al. 2002). Longer distance dispersal is commonly achieved by ballooning, a process in which the larvae lowers itself on a strand of silk from a leaf and is carried by the wind to adjacent plants (Zalucki et al. 2002). Andow (2002) reported that neonates silk from substrates to disperse and to escape disturbances, such as predators. Ross and Ostlie (1990) found that dispersal is greatest in the first 48 hours after eclosion, so neonate behavior is important in determining how a refuge should be established within a Bt cornfield. Determining dispersal rates and distances will be necessary in deciding the arrangement and size of refuge areas (Engels et al. 2008).

Ross and Ostlie (1990) found that approximately 50% of ECB larvae abandon the natal plant and 90% of those larvae disperse 43-47 cm from the infested plant within a non-Bt corn field. Field exposure to toxins present in Bt corn seems to increase the likelihood of ECB larval dispersal between plants (Davis and Onstad 2000). Cohen et al. (1987) and Schiff et al. (1989) suggested that insects may get physiological feedback from a toxic or nutritionally inadequate food source, which then causes them to show increased movement in search of a better food source. ECB dispersal experiments conducted by Goldstein et al. (2010) indicated that most of ECB

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neonates are able to detect the Bt endotoxins when exposed to the plant for 24 h which elicits higher rates of plant abandonment when compared to neonate dispersal behavior on non-Bt plants.

Observations describing high rates of ECB larval dispersal between Bt and non-Bt corn has created significant concern regarding the deployment of seed mixtures as a resistance management strategy (Mallet and Porter 1992, Schnepf et al. 1998, Prasifka et al. 2009, Goldstein et al. 2010). ECB larvae moving from plant to plant may receive a lower dose of Bt toxins, increasing the likelihood of heterozygote survival and potentially accelerating the development of resistance (Mallet and Porter 1992). The formation of behavioral resistance in ECB populations could also hasten if larvae are successfully able to disperse and develop on non-Bt corn (Goldstein et al. 2010). However, with the development of pyramid Bt corn that requires only a 5% refuge, the likelihood of a larvae dispersing from Bt corn and reaching a non-Bt corn plant is greatly reduced compared to corn with a single Cry protein Bt trait.

Furthermore, in order for the larvae to develop partial resistance to Bt toxins, it would be assumed that the larvae feed on the Bt corn prior to dispersal and subsequent survival. However, minimal data is available on how much, if any, feeding takes place before dispersal. Goldstein et al. (2010) suggested that it is unlikely that ECB can detect Bt with receptors on its tarsi, maxillary palpi, or integument, but there is little

documentation available to state this with certainty. Additional knowledge of neonate

ECB feeding behavior on Bt corn is needed to better understand dispersal and survival,

and therefore to determine if the seed mixture strategy is a viable control option.

The goal of this study was to better understand the behavioral characteristics of

the neonate ECB on Bt corn hybrids and its potential role in the development of

resistance to Bt crops. The specific objectives were to 1) compare dispersal behavior

of neonates on Bt field corn, non-Bt field corn, and non-Bt sweet corn; 2) assess

neonate responses on Bt lines associated with a pyramid hybrid being developed for

commercial use; 3) compare the silking response on Bt and non-Bt under light wind

conditions; and 4) characterize and compare the degree of neonate feeding on Bt and

non-Bt corn during the first 48 hours after eclosion. I hypothesized that ECB larvae

abandon Bt corn plants at a higher rate than they abandon non-Bt corn plants, and that

dispersal rates will be highest on a Bt pyramid hybrid when compared to other Bt

hybrid lines. I expected to see higher rates of larval abandonment on Bt corn under

wind conditions when compared to non-Bt corn or Bt corn that is not exposed to wind.

Finally I expected to see a higher degree of neonate feeding on non-Bt corn plants than

on Bt corn plants. The information obtained through this project will be useful in

developing the most effective insect resistance management (IRM) strategies.

MATERALS AND METHODS

Insect Specimens

Insects for this study were obtained from the USDA Corn Institute Research Laboratory located in Ames, Iowa. For establishing a new colony there each year, ECB egg masses were collected from the field in Iowa by the late summer or early fall. The larvae were raised in the laboratory, where they pupated, mated, and laid more egg masses for the next generation. This cycle in the laboratory was repeated throughout the year in Iowa for about 12 generations. To prepare for this study, ECB pupae were shipped from the Iowa laboratory and kept in growth chambers (Percival Scientific, Perry, IA) at the University of Delaware (UD) Entomology laboratories. The pupae were sent in cardboard rings, from which they were removed and placed in cages. Once the adults emerged, a male and female were paired together in a 0.5 liter paper cylindrical carton and left to reproduce. Females laid eggs on wax paper that was placed at the top of the carton. The wax paper with egg masses was removed and placed into a plastic Ziploc bag (S.C. Johnson and Sons, Inc. Racine, WI) with a moistened cotton ball. The pupae, adults, and eggs were kept in two growth chambers – one maintained at 20°C and another at 25°C – in order to stagger the emergence of the adults and larvae. The growth chambers were set for 16 h of light and 8 h of darkness per day and relative humidity was maintained between 50% and 60%.

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Neonates were used in all bioassays because they are more mobile and are mostly responsible for accepting the host when compared to other life stages (Zalucki et al. 2002).

Plants

The Bt corn hybrid used in the field study was a Pioneer Hi-Bred brand (Johnston, IA) stacked pyramid. The traits that were incorporated in the stacked pyramid corn hybrid include Herculex XTRA, DAS 591227-7 × TC1507 (Cry34Ab1 + Cry35Ab1 + Cry1F) combined with YieldGard® Corn Borer, MON810 (Cry1Ab) (See Appendix for Bt products). A non-Bt near isoline hybrid from the same parent background that genetically matched the Bt variety provided by Pioneer Hi-Bred was used to represent corn plants possessing native resistance traits to insects, which is acquired through plant breeding that is consistent with non-transformed commercial field corn varieties. Both corn treatments also contained the Roundup Ready® gene (event NK603) for tolerance to glyphosate herbicide. 'Silver Queen' sweet corn (non-Bt) was utilized in the field experiment to represent a plant highly susceptible to ECB and lacking the native resistance traits derived through many years of traditional corn breeding that are found in commercial field corn varieties. For the laboratory experiments (including wind and feeding tests), corn plants were grown in the greenhouse in 3 gallon pots until the six-leaf stage, watered daily, and not subject to

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any insecticide applications. The Bt corn treatments used in the laboratory

experiments included Pioneer Hi-Bred brand (Johnston, IA) corn hybrids Herculex 1,

TC1507 event (Cry1F), Bt corn; YieldGard Corn Borer, MON810 event (Cry1Ab), Bt

corn; and pyramid TC1507 × MON810 (Cry1F x Cry1Ab). All three hybrids

originated from the same parent lines. Consequently, they were nearly genetically

identical except for the Bt traits. A non-Bt near isoline hybrid from the same parent

background that genetically matched the Bt varieties provided by Pioneer Hi-Bred was

also used in the laboratory experiments.

Plant Abandonment in the Field

A preliminary study was conducted in corn fields located on the UD Farm in

Newark, DE from June 25, 2008 to July 24, 2008 to evaluate neonate ECB dispersal

behavior. Two treatments of corn were used in this study: a Bt pyramid combining

MON810 and Roundup Ready®, event NK603; and non-Bt 'Silver Queen' sweet corn.

The two treatments of corn were planted in separate fields on the UD Farm. An ECB

egg mass was placed a plant from each treatment and applied to the underside of the

leaf with a droplet of water. After the first larva emerged from the egg mass,

observations on the behavioral characteristics were made for the first hour. The

behavioral characteristics that were categorized were the number of larvae on the leaf,

at the leaf axil, that silked off the leaf, that were predated, and that silked but did not

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leave the leaf (silking attempts). Larvae characterized on the leaf included those on

the blade and sheath, but not the leaf axil. Multiple trials were conducted during a day

and a total of ten replicates were generated for pyramid corn and eleven replicates

were generated for sweet corn in this study. The field bioassay was also performed in

corn fields located on the UD Farm. This experiment was conducted from July 14,

2009 to July 30, 2009. During this time period, the average daytime temperature was

23.3°C and the average wind speed was 1.7 mph (DEOS 2009). ECB egg masses in

the blackhead stage were transported to the field in a cooler and placed on three

treatments of corn: the stacked pyramid DAS 59122-7 $\times$ TC1507 $\times$ MON810 Bt corn,

non-Bt near isoline, and non- Bt 'Silver Queen' sweet corn. The stacked pyramid and

non-Bt near isoline corn used in this bioassay were already established in a field

experiment conducted for Pioneer Hi-Bred evaluating the efficacy of Bt corn deployed

with a blended refuge against ECB. The Pioneer Hi-Bred experimental design

involved five treatments in a randomized complete block design with three

replications where each plot was planted in four rows of which only the middle two

rows were used (Figure 1). For the field bioassay conducted in this study, four plants

were randomly assigned to each of the two treatments in the first and fourth rows of

each plot that were not used (Figure 1). The 'Silver Queen" sweet corn was planted in

a field adjacent to the Pioneer Hi-Bred research field. Four sweet corn plants were

assigned on the border of the field nearest the Pioneer Hi-Bred research field. Over

the two-week time period, to carry out the experiment, stages of corn development

varied from the late whorl stage to the reproductive silking stage (Mason et al. 1996).

For each replicate (trial), an egg mass was placed on a plant from each treatment by

applying it to the underside of the leaf with a drop of water. The leaf chosen varied

from the sixth leaf to the eighth leaf, depending on the development stage of the corn

plant. After the first larva emerged from each egg mass, observations on the

behavioral characteristics were made for four hours and then the larvae were removed

from the plant and destroyed. The behavioral characteristics that were categorized

were the number of larvae on the leaf, at the leaf axil, that silked off the leaf, that were

predated, and that silked but did not leave the leaf (silking attempts). Only one trial

could be accomplished during a day and a total of twelve replicates were generated for

the study.

Plant Abandonment in the Laboratory

ECB egg masses in the blackhead stage were placed simultaneously on four

treatments of corn in the six-leaf stage: non-Bt near isoline corn, TC1507 hybrid corn,

MON810 hybrid corn, and a pyramid hybrid corn combining TC1507 and MON810.

The corn plants were in 3 gallon pots placed on the floor of the laboratory without

exposure to wind movement. The average temperature in the laboratory was 22.2°C

and relative humidity ranged from 40% to 70%. A trial consisted of one plant

represented in each of the four treatments. For each treatment, one egg mass was

applied to the underside of the fifth leaf with a drop of water. Behavioral

characteristics were observed and recorded for four hours after eclosion of the first

larva for each cohort. Larvae were removed from the plants after the four hour

observation period. The response data included the number of larvae on the leaf, at the

leaf axil, that silked off the leaf, and that silked but did not leave the leaf (silking

attempts). Thirteen replicates were generated for non-Bt near isoline and the pyramid

hybrid, and eleven replicates were generated for TC1507 and MON810. Results from

two trials were not recorded for both TC1507 and MON810 treatments because larvae

did not emerge from the egg mass, possibly due to the eggs not being fertile.

Influence of Wind on Silking Behavior

This bioassay was performed in a wind tunnel under conditions of constant

wind flow to assess differences in silking behavior between Bt and non-Bt corn. The

average temperature in the wind tunnel room was approximately 24.0°C (ranged from

22.9°C to 25.4°C) and the relative humidity ranged from 33% to 71%. Two corn types

in the six-leaf stage were used: MON810 and non-Bt near isoline corn plants. Four

treatments were compared: non-Bt isoline exposed to wind, non-Bt isoline not exposed

to wind, MON810 exposed to wind, and MON810 not exposed to wind. One plant

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from each corn type was placed in front of the wind tunnel and was exposed to a

constant air flow of 70 cm/s (1.6 mph). A mesh screen was placed over the opening of

the wind tunnel to catch any larvae that silked off the plants, and the plants were

placed within 30 cm from the mesh screen to simulate realistic plant distances within

corn field rows (Pilcher et al. 1997). At the same time, another plant from each corn

type was placed away from the wind tunnel such that the plant was not exposed to air

flow. The plants used were randomly assigned a treatment for each replication. The

plants were arranged so that the leaves from each plant did not touch and larvae could

not walk from plant to plant. One egg mass was placed on the underside of the fifth

leaf with a drop of water for each treatment. Behavioral characteristics were observed

and recorded for four hours after eclosion of the first larva and the larvae were

removed from the plants at the end of four hours. The response data included the

number of larvae on the leaf, at the leaf axil, that silked off the leaf, and that silked but

did not leave the leaf (silking attempts). Eleven replicates were generated for each

treatment.

Changes in Neonate Feeding Behavior over Time

This bioassay was conducted in the laboratory to determine feeding rates for

neonate ECB on MON810 Bt and non-Bt near isoline corn in the sixth-leaf stage. One

ECB egg mass was placed on the underside of the fifth leaf with a drop of water for

each treatment. Larvae were exposed to the plants for four different time intervals: 6 h, 12 h, 24 h, and 48 h. There were 10 replicates for each trial. One trial consisted of a block which had one plant representing each corn type that was randomly assigned each time interval for a total of eight plants in the trial. Clear plastic bags were tied around the stalk of the plants to ensure larvae did not leave the plant. At the end of each time interval, each plant was dissected and larvae were removed from the plant with a small paintbrush. Larvae were categorized by whether they were found on the leaf, in the whorl, or on the bag. Once separated, the larvae were mounted onto microscope slides. Karo® (ACH Food Companies, Inc., Memphis, TN) light corn syrup diluted with water was used as a mounting medium (Johansen 1940) and covered with a coverslip. Using a compound microscope, larvae were examined to determine if there was evidence they had fed. Evidence of feeding was determined by the presence of chlorenchyma and tracheary elements from the plant in the gut of the insect (Figure 2). Chlorenchyma is parenchyma specialized for photosynthesis and has an abundance of chloroplasts; and tracheary elements are the conducting cells of the xylem (Evert 2006). A rating system was constructed to estimate the amount of plant tissue present in the gut of each larva (Table 1).

Data Analysis

The data collected were compared for behavioral differences between the treatments. If there were more than two treatments being compared, significance was tested for by using a one-way analysis of variance (ANOVA) using Minitab. Significant differences between treatments were separated using Fisher's Least Significant Difference (LSD) test using Minitab. For the bioassay on changes in neonate feeding behavior over time, log transformations were used to normalize the data.

RESULTS

Plant Abandonment in the Field

For the preliminary study conducted in the field, the average number of larvae that hatched from each egg mass on sweet corn was 30.6 and on the MON810 × NK603 Bt corn was 28.8. There was a significantly higher percentage of larvae that silked off the plant on the MON810 × NK603 Bt corn compared to the sweet corn (one-way ANOVA: $F = 8.42$; df = 1, 20; $P = 0.009$) (Table 2, Figure 3). There was also a significantly higher frequency of silking attempts (number of silking attempts/total number of hatched larvae) on the MON810 × NK603 Bt corn compared to the sweet corn (one-way ANOVA: $F = 9.10$; df = 1, 20; $P = 0.007$) (Figure 4).

For the plant abandonment bioassay conducted in the field, the average number of larvae that hatched from each egg mass was 24.4 on sweet corn, 25.9 on non-Bt near isoline, and 26.7 on DAS59122-7 × TC1507 × MON810 Bt corn. There was a significant difference for the mean percentage of larvae that silked off the plant on sweet corn compared to DAS 59122-7 × TC1507 × MON810 Bt corn and non-Bt near isoline (one-way ANOVA: $F = 5.78$; df = 2, 35; $P = 0.007$) (Table 3). The mean percentage of larvae silking off the plant was lowest for sweet corn (1.8%) and highest for non-Bt near isoline (16.2%) (Table 3, Figure 5). The mean proportion of larvae that silked off the plant was lower on the DAS 59122-7 × TC1507 × MON810 Bt corn

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(11.9%) (Table 3, Figure 5) when compared to the non-Bt near isoline, however, this difference was not significant. There was also a significant difference for the frequency of silking attempts on sweet corn compared to DAS 59122-7 × TC1507 × MON810 Bt corn and non-Bt near isoline (one-way ANOVA: F = 4.94; df = 2, 35; P = 0.013) (Figure 6). The frequency of silking attempts was lowest for sweet corn (0.15), followed by DAS 59122-7 × TC1507 × MON810 Bt corn (0.38), and was highest for non-Bt near isoline (0.46) (Figure 6). A significantly higher percentage of larvae were predated on sweet corn (10.6%) and non-Bt near isoline (5.3%) compared to DAS 59122-7 × TC1507 × MON810 Bt corn (1.7%) (one-way ANOVA: F = 7.64; df = 2, 35; P = 0.002) (Table 3, Figure 5). Predators that were observed in the field included the minute pirate bug and lady beetles. Although the difference was not significant between treatments, a greater percentage of larvae were found in the leaf axil compared to other parts of the leaf for all treatments (Table 3, Figure 5).

Plant Abandonment in the Laboratory

The average number of larvae that hatched from each egg mass was 27.5 on non-Bt near isoline, 31.4 on TC1507, 24.5 on MON810, and 30.5 on TC1507 × MON810 Bt corn. MON810 had the highest mean percentage of larvae that silked off the plant (2.9%) when compared to non-Bt near isoline (0.7%), TC1507 (0.7%), and TC1507 × MON810 Bt corn (0.8%) (Table 4, Figure 7). However, none of these were

significant. There was a significantly lower frequency of silking attempts on non-Bt near isoline (0.35) and TC1507 (0.40) compared to TC1507 × MON810 Bt corn (0.53); and there was a significantly lower frequency of silking attempts on non-Bt near isoline, TC1507, and TC1507 × MON810 compared to MON810 (0.77) (one-way ANOVA: $F = 11.46$; df = 3, 47; $P \leq 0.001$) (Figure 8). I analyzed the difference for the frequency of silking attempts for each hour within a four hour period between treatments (Figure 9). By the second hour there was a significantly higher frequency of silking attempts on MON810 (0.23) and TC1507 × MON810 Bt corn (0.19) compared to non-Bt near isoline (0.11) and TC1507 (0.12) (one-way ANOVA: $F = 3.31$; df = 3, 47; $P = 0.029$). For the third hour there was a significantly higher frequency of silking attempts on MON810 (0.28) compared to non-Bt near isoline (0.11), TC1507 (0.13), and TC1507 × MON810 Bt corn (0.18) (one-way ANOVA: $F = 6.20$; df = 3, 47; $P = 0.001$). For the fourth hour there was a significantly lower frequency of silking attempts on non-Bt near isoline (0.06) compared to TC1507 (0.10), TC1507 × MON810 (0.10), and MON810 (0.15) (one-way ANOVA: $F = 3.87$; df = 3, 47; $P = 0.015$). Although the difference was not significant between treatments, there was a higher mean percentage of larvae found on the leaf compared to in the leaf axil for all treatments (Table 4, Figure 7).

Influence of Wind on Silking Behavior

The average number of larvae that hatched from each egg mass was 27.7 on non-Bt near isoline with wind, 20.8 on non-Bt near isoline with no wind, 27.7 on MON810 with wind, and 26.0 on MON810 with no wind. There was a significantly greater mean proportion of larvae that silked off the plant between MON810 with wind compared to the other three treatments (one-way ANOVA: $F = 11.6$; df = 3, 43; $P \leq 0.001$) (Table 5). MON810 with wind had the greatest mean percentage of larvae that silked off the plant (15.1%), and non-Bt near isoline with no wind had the least (0.6%) (Table 5, Figure 10). Although the difference was not significant, I also found that non-Bt near isoline with wind had a higher mean percentage of larvae that silked off the plant (3.7%) than MON810 with no wind (2.1%) (Table 5, Figure 10). There was a significantly higher frequency of silking attempts on MON810 with no wind compared to the other three treatments (one-way ANOVA: $F = 5.64$; df = 3, 43; $P = 0.003$) (Figure 11).

Changes in Neonate Feeding Behavior over Time

After 6 hours, for both non-Bt near isoline and MON810, slightly more than half of the larvae had left the corn plant and were found on the bag (Table 6). A significantly higher mean percentage of larvae had fed (i.e. had plant material in the gut, Figure 2) that were found on the leaf on non-Bt near isoline (7.1%) compared to

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MON810 (0.4%) (Table 6). After 12 hours, a significantly higher mean percentage of larvae were found on the leaf on non-Bt near isoline (35.5%) compared to MON810 (19.2%) (Table 6). There was also a significantly higher mean percentage of larvae found on the bag on MON810 (65.0%) than on non-Bt near isoline (51.3%) (Table 6). A significantly higher mean percentage of larvae had fed that were found on the leaf on non-Bt near isoline (18.9%) compared to MON810 (3.3%) (Table 6). After 24 hours, there was a significantly higher mean percentage of larvae found on the leaf on non-Bt near isoline (56.2%) compared to MON810 (20.8%) (Table 6). There was also a significantly higher mean percentage of larvae found on the bag on MON810 (66.0%) compared to non-Bt near isoline (30.9%) (Table 6). A significantly higher mean percentage of larvae had fed that were found on the leaf on non-Bt near isoline (45.4%) compared to MON810 (11.7%) (Table 6). After 48 hours, a significantly higher mean percentage of larvae were found on the leaf on non-Bt near isoline (48.2%) compared to MON810 (27.4%) (Table 6). There was a significantly higher mean percentage of larvae found in the whorl on non-Bt near isoline (12.2%) compared to MON810 (0.8%) (Table 6). There was also a significantly higher mean percentage of larvae found on the bag on MON810 (49.9%) compared to non-Bt near isoline (23.1%) (Table 6). A significantly higher mean percentage of larvae had fed that were found on the leaf on non-Bt near isoline (36.3%) compared to MON810

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(16.5%) (Table 6). A significantly higher mean percentage of larvae had fed that were

found in the whorl on non-Bt near isoline (11.4%) compared to MON810 (0.0%)

(Table 6). There was also a significantly higher mean percentage of larvae that had fed

that were found on the bag on MON810 (26.6%) compared to non-Bt near isoline

(12.7%) (Table 6). Significant differences were found between treatments for the

mean quantity of plant tissue in the gut of larvae that had fed that were found on the

leaf at 6 h ($p = 0.005$), 12 h ($p \leq 0.001$), 24 h ($p \leq 0.001$), and 48 h ($p \leq 0.001$) (Figure

12). The mean quantity of plant tissue in the gut of larvae that had fed that were found

on the leaf was significantly higher in larvae that fed on non-Bt near isoline compared

to MON810 at all four time intervals (Figure 12).

DISCUSSION

<u>Plant Abandonment in the Field</u>

The findings from this experiment suggest that the larvae are significantly

more likely to silk off a plant, or attempt to silk off a plant, that has Bt or a genetically

similar native resistant non-Bt near isoline hybrid than the sweet corn plant which

likely has much less native resistance (Mason et al. 1996). Although the difference

was not significant, I did not expect to observe a higher percentage of larvae silking on

non-Bt near isoline than on Bt corn. Previous field and laboratory observations

suggest that exposure to toxins present in Bt corn seems to increase the likelihood of

ECB larval abandonment compared to non-Bt corn (Davis and Onstad 2000, Goldstein

et al. 2010). It is important to note that the Bt corn used in the field experiment

conducted in this study had a different set of Cry and herbicidal genes compared to Bt

corn used in previous experiments. Although non-Bt near isoline does express

traditional resistance traits, the endotoxins expressed by Bt crops are expected to act as

a significantly stronger deterrent against pest damage, and therefore are expected to

elicit behaviors leading to plant abandonment in response. The time spent traveling

between plants within the Pioneer Hi-Bred plot and between the Pioneer Hi-Bred and

sweet corn plots may have resulted in missed silking observations and could account

for the unexpected results. Experimental field plots set up so there is less travel time

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between treatments may reduce the experimental error that was involved in this design.

The findings from the field bioassay also suggest that predation is greater in sweet corn fields than in Bt fields. One explanation for this observation may be that since a greater density of larvae are able to survive in the sweet corn, more predators could be attracted to sweet corn when compared to Bt corn. This finding could have important implications for a reduction in refuge size requirements in Bt fields. If the refuge area is reduced, it may support a smaller ECB population, and therefore could reduce the abundance of natural enemies that are available for control of pests in the field.

Observations made for neonate dispersal behavior in the field bioassay also suggest there was a higher mean percentage of larvae at the leaf axil than on the other parts of the leaf for all three treatments. Andow (2002) reported that predators are more effective preying on the neonates when they are exposed on leaf surfaces. The findings from my bioassay may indicate that within the first four hours of emergence, larvae either silk off the plant or disperse to the leaf axil for shelter from predators and desiccation.

Plant Abandonment in the Laboratory

I observed relatively low neonate dispersal across treatments for the first four hours after eclosion in this laboratory bioassay. I did not expect to observe such low dispersal rates when compared to the higher dispersal rates that were observed in the field. Previous laboratory bioassays conducted under conditions with light air movement by Goldstein et al. (2010) found that the proportion abandoning the plant was significantly higher for Bt than non-Bt corn. I hypothesize that the absence of wind in this bioassay resulted in a lower proportion of larvae silking off the plant than was observed in the field or would have been in a laboratory bioassay conducted under light wind conditions. I also found there was a significantly higher frequency of silking attempts on MON810 compared to TC1507 (Table 4). This finding may suggest that the Cry proteins expressed in MON810 (Cry1Ab) have a greater effect on neonate ECB dispersal behavior than the Cry proteins expressed in TC1507 (Cry1F). Buntin (2008) evaluated the efficacy of MON810 and TC1507 for management of fall armyworm and corn earworm in central Georgia. He found that although both Bt events reduced whorl infestation and damage by fall armyworm, TC1507 provided greater protection from whorl injury compared to MON810 under severe fall armyworm infestations. He also found that MON810 usually had less ear infestation by corn earworm than susceptible hybrids, whereas TC1507 usually did not reduce ear

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infestations. These findings suggest that the efficacy of Bt events vary not only between insect pests, but also in terms of the protection provided by Bt events at different locations on the corn plant. Therefore, it is possible that MON810 and TC1507 could provide different behavioral responses as well as levels of control against ECB damage. Further research comparing the efficacy of these two Bt events could have important implications for the use of stacked pyramid hybrids combining MON810 and TC1507. If one event provides significantly less protection against ECB damage compared to the other, there may be less selection pressure against resistance developing in ECB populations.

The observations recorded for silking attempts showed that the frequency of silking attempts for all four treatments consistently was the highest at the second and third hours, and decreased at the fourth hour (Figure 9). This may suggest that after the third hour a percentage of the larvae exhibiting dispersal behavior are switching to local leaf exploration in search of food. However, there was no visible evidence of leaf feeding on any of the four treatments. This bioassay offered significant insights into neonate ECB dispersal behavior for the first four hours after eclosion on both Bt and non-Bt corn. The results suggest that silking behavior may be an indicator for host selection when comparing non-Bt and Bt corn.

Influence of Wind on Silking Behavior

The findings in this laboratory bioassay support the hypothesis that light wind facilitates larvae silking off the plant. I hypothesized that there would be a significantly higher percentage of larvae silking off the plant on MON810 with wind compared to MON810 with no wind due to the effect of wind on silking behavior. I also hypothesized that there would be a higher frequency of silking attempts on MON810 with no wind compared to MON810 with wind because the lack of wind reduced the proportion of larvae that were able to successfully silk off the plant. The data supported these two hypotheses.

Wind facilitated larval dispersal is well documented in some lepidopteran taxa, such as Lymantriidae (i.e. gypsy moths), in which high rates of ballooning and aerial transportation have been cited as important factors in long-distance dispersal and spreading infestations (Bell et al. 2005, Tobin and Blackburn 2008). Mott (1963) described how first instar spruce budworm (Lepidoptera: Tortricidae) larvae drop down from a plant surface and then attempt to become airborne through suspended ballooning in response to turbulence. Neonate evergreen bagworms (Lepidoptera: Psychidae) have also been observed to disperse by dropping on a strand of silk and ballooning on the wind (Moore and Hanks 2004). I believe that wind is an important factor to include in studies on ECB larval dispersal and silking behavior under

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controlled conditions. Utilizing wind in laboratory experiments will also provide results that are more consistent and comparable to conditions in the field.

The observations made on neonate dispersal behavior are important in resistance management, specifically where and how refuge plants are sowed within a Bt corn field. Larvae hatching on Bt plants are more likely to silk off the plant and may end up on nearby non-Bt plants. This is more likely in a mixed seed than in a structured refuge system. Therefore, if the larvae are successful at finding more suitable host plants after exposure to toxins in Bt corn, it could allow for behavioral resistance to evolve more quickly in ECB populations. However, a reduction in refuge proportion within a Bt corn field could make the likelihood of a larva moving from a Bt to a non-Bt plant less likely due to the lower percentage of non-Bt plants. Therefore, the development of pyramid Bt corn and the associated reduction in the required proportion of refuge plants could provide support for the implementation of a mixed plant refuge. Even with high observed dispersal rates in ECB neonates, the likelihood of a larva dispersing from a Bt to a non-Bt plant and surviving is greatly reduced in a mixed seed refuge system that would require a smaller percent refuge, which would delay the development of resistance in ECB populations.

Changes in Neonate Feeding Behavior over Time

In this laboratory bioassay, I observed that slightly more that 50% of the larvae dispersed off the plant and were found on the bag for both treatments. It is also interesting to note that the mean proportion of larvae that had evidence of feeding that were found on the bag was 1.5% and 1.8% for non-Bt isoline and MON810, respectively. This indicates that more than 95% of the larvae for both non-Bt isoline and MON810 had left the plant and were found on the bag with no evidence of feeding based on plant material in the gut. This finding was unexpected and challenges the notion that feeding precludes the choice to remain on the natal plant or disperse. It is possible that a percentage of individuals in an egg mass, which contain anywhere from 15 to more than 30 eggs, are genetically programmed to disperse from the natal plant after eclosion without feeding in search of a suitable host plant. The larvae would be less likely to encounter competition from kin which could increase the likelihood of survival for an individual larva. The level of larval infestation on a plant that can cause economic damage can be as low as two mature larvae per plant (Mason et al. 1996). This indicates that the ability for a plant to support a full compliment of larvae from an egg mass is limited. Consequently, there is a selective advantage if some of the larvae resulting from an egg mass leave the natal plant to avoid competition and become established on an alternative plant.

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Results demonstrating a significantly higher percentage of larvae that had evidence of feeding that were found in the whorl after 48 hours on non-Bt near isoline compared to MON810 suggest that larvae are more likely to establish and begin feeding in the whorl on non-Bt isoline than on MON810. Previous studies found that significantly more neonates were recovered from the whorl of non-Bt than from Bt corn (Goldstein et al. 2010). This suggests that larvae that disperse to the whorl are likely to be accepting the host. In the present study, after 48 hours, there was also a significantly higher percentage of larvae that had evidence of feeding that were found on the bag on MON810 compared to non-Bt near isoline. This finding suggests larvae that have fed on the plant are more likely to abandon the plant after feeding on MON810 than on non-Bt isoline. Furthermore, my findings suggest that the amount of plant material consumed is significantly higher on non-Bt isoline compared to MON810. Davis and Coleman (1997) observed neonates changing their consumption rates of Bt corn over time such that there was initially high leaf consumption followed by little to no leaf consumption. Although I did not observe a reduction in consumption over time, the findings from this bioassay suggest that larvae are consuming less plant material on Bt compared to non-Bt plants. These findings could be useful for determining effective strategies in resistance management. If larvae are able to detect Bt toxins after feeding on plants for 24 h and reduce their consumption

over time, they may not ingest enough toxin, and therefore could disperse to a non-Bt

plant which could accelerate resistance development in ECB populations if these

larvae carry a trait for resistance.

In general, my findings suggest that dispersal is relatively high based on

neonates recovered from the bag for both treatments within the first 24 hours. After

24 hours, a higher percentage of larvae are likely to establish on non-Bt isoline, while

dispersal from MON810 remains high. Tang et al. (2001) reported similar findings for

ECB dispersal experiments comparing Bt and non-Bt corn for a 72-hour period. They

found that when the release host was a Bt plant, most larval movement off the Bt plant

occurred during the first 48 hours with little change of movement between 48 and 72

hours. When the release host was a non-Bt plant, all larval movement occurred during

the first 24 hours with little change of movement between 24 and 72 hours. Most

movement onto the second plant occurred within the first 24 hours of the release with

little movement occurring afterwards (Tang et al. 2001).

Overview of the Studies

Overall, I found that neonate dispersal in the field was higher on non-Bt isoline

and DAS 59122-7 $\times$ TC1507 $\times$ MON810 Bt corn when compared to sweet corn, with

no significant differences in dispersal between non-Bt isoline and DAS 59122-7 $\times$

TC1507 $\times$ MON810 Bt corn. In the laboratory, I found that differences in dispersal

behavior between Bt and non-Bt corn were not expressed until the second hour after

eclosion. I also observed relatively low neonate dispersal for both Bt and non-Bt corn

when wind was not included in the experimental design. The results obtained in the

laboratory bioassay conducted under light wind conditions suggest that neonates

hatching on MON810 corn are significantly more likely to disperse than those

hatching on non-Bt near isoline corn. My findings from the feeding bioassay support

the hypothesis that feeding behavior is significantly different between non-Bt near

isoline and MON810 corn and detection of the Bt toxin through feeding increases the

likelihood of dispersal from a Bt plant. However, approximately 50% of the larvae are

likely to disperse from their natal plant, whether it is a Bt plant or non-Bt plant, with

no evidence of feeding. It will be necessary to explore the relationship between larval

feeding and dispersal on Bt to understand movement between Bt and non-Bt plants

and the likelihood of survival after ingesting Bt toxins. Previous laboratory findings

suggest that late-instar larvae can move from non-Bt corn to Bt corn and survive to

adulthood (Walker et al. 2000, Huang et al. 2002), but there is little evidence of this

occurring in the field. Further research to evaluate these unknown differences in

behavior will be necessary to determine the efficacy of a mixed refuge strategy and the

associated risk of resistance development in ECB populations. These results could aid

in understanding how ECB larvae detect Bt and how detection affects dispersal

behavior over time. The dispersal behavior of neonate ECB will be important to

consider and understand in establishing a refuge and delaying the development of

resistance in ECB populations. These findings may not only be useful for ECB

resistance management, but may also apply to other species that behave similarly

under Bt selection pressure.

APPENDIX

Appendix. Some Bt products available for corn insect protection. (Assembled by C. E. Mason)

Brand	Company	Event	Transgenic Component	Target Pests[*]	Herbicide Tolerant
Agrisure CB	Syngenta	BT11	Cry1Ab	ECB	No
YieldGard Corn Borer	Monsanto	MON810	Cry1Ab	ECB	No
YieldGard Rootworm	Monsanto	MON863	Cry3Bb1	CRW	No
Herculex 1	Dow	TC1507	Cry1F	ECB, BCW, FAW	Yes
Herculex RW	Dow	DAS591227-7	Cry34Ab1 + Cry35Ab1	CRW	No
Herculex Extra	Dow	DAS59122-7 x TC1507	Cry34Ab1 + Cry35Ab1 + Cry1f	CRW, ECB, BCW, FAW	Yes
YieldGard Plus	Monsanto	MON863 x MON810	Cry3Bb1 + Cry1Ab	CRW, ECB	No
YieldGard VT RW	Monsanto	MON88017	Cry3Bb1	CRW	Yes
YieldGard VT Plus	Monsanto	MON88017 x MON810	Cry3Bb1 + Cry1Ab	CRW, ECB	Yes
Agrisure RW	Syngenta	MIR604	mCry3A	CRW	No
Agrisure 3000 CB/RW	Syngenta	MIR604 x Bt11	mCry3A + Cry1Ab	ECB, CRW	Yes
	Monsanto	MON89034	Cry1A.105 + Cry2Ab2	ECB, SWCB, FAW, CEW	No
	Monsanto	MON89034 x MON88017	Cry1A.105 + Cry2Ab2 + Cry3Bb1	ECB, SWCB, FAW, CEW, CRW	Yes
Agrisure 3100	Syngenta	Bt11 x MIR162 x MIR604	Cry1Ab + Vip3Aa20 + mCry3A	Many lepidopterans & rootworms	Yes
SmartStax	Monsanto & Dow	MON89034 x TC1507 x MON88017 x DAS59122-7	Cry1A.105 + Cry2Ab2 + Cry1F + Cry3Bb1	ECB, SWCB, FAW, CEW, BCW, CRW	Yes

[*] ECB – European corn borer, CRW – corn rootworm, BCW – black cutworm, FAW – fall armyworm, ECW – corn earworm, SWCB – southwestern corn borer.

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TABLES AND FIGURES

Table 1.   Rating system to quantify the amount of plant tissue in the gut of neonate ECB for the feeding behavior bioassay.

Rating	Description
0	No plant tissue in the gut
1	Very little plant tissue in the gut
2	< 25% plant tissue in the gut
3	25% - 50% plant tissue in the gut
4	50% - 75% plant tissue in the gut
5	75% - 100% plant tissue in the gut

Table 2.   Mean percentage of larvae recorded for the preliminary study conducted in the field during the first hour after eclosion for each behavior outcome for non-Bt sweet corn and pyramid (MON810 × NK603) Bt corn.

	Mean ± SEM		ANOVA Statistical parameter		
Outcome	Sweet corn (n = 11)	MON810 × NK603 (n = 10)	df	F	P
At leaf axil	41.7 ± 6.7a	46.0 ± 3.6a	1, 20	0.29	0.594
At leaf	39.6 ± 7.9a	30.0 ± 4.2a	1, 20	1.10	0.308
Predated	0.3 ± 0.3a	2.7 ± 1.6a	1, 20	2.50	0.130
Silked off	1.0 ± 0.5b	6.8 ± 2.0a	1, 20	8.42	0.009
Unaccounted	17.6 ± 3.5a	14.8 ± 3.0a	1, 20	0.37	0.552

Means in row followed by the same letter are not significantly different (Fisher LSD, $P \leq 0.05$).

Table 3. **Mean percentage of larvae recorded for the plant abandonment bioassay in the field during the first four hours after eclosion for each behavior outcome for sweet corn, stacked pyramid (DAS 59122-7 × TC1507 × MON810) Bt corn, and non-Bt near isoline.**

Outcome	Mean ± SEM			ANOVA Statistical parameter		
	Sweet corn (n = 12)	Non-Bt near-isoline (n = 12)	Pyramid (n = 12)	df	*F*	*P*
At leaf axil	66.8 ± 3.1a	57.8 ± 4.1a	61.4 ± 2.9a	2, 35	1.78	0.184
At leaf	5.2 ± 2.7a	6.9 ± 2.8a	5.4 ± 2.5a	2, 35	0.12	0.885
Predated	10.6 ± 2.0a	5.3 ± 1.6a	1.7 ± 1.0b	2, 35	7.64	0.002
Silked off	1.8 ± 0.9b	16.2 ± 4.3a	11.9 ± 3.0a	2, 35	5.78	0.007
Unaccounted	17.3 ± 2.7a	13.9 ± 2.6a	19.6 ± 3.2a	2, 35	1.02	0.373

Means in row followed by the same letter are not significantly different (Fisher LSD, $P \leq 0.05$).

Table 4. **Mean percentage of larvae recorded for the plant abandonment bioassay in the laboratory during the first four hours after eclosion for each behavior outcome for non-Bt near isoline, TC1507, MON810, and pyramid (TC1507 × MON810) Bt corn.**

Outcome	Mean ± SEM				ANOVA Statistical parameter		
	non-Bt isoline (n = 13)	TC1507 (n = 11)	MON810 (n = 11)	Pyramid (n = 13)	df	*F*	*P*
At leaf axil	35.1 ± 5.3a	34.6 ± 6.2a	26 ± 5.6a	24.8 ± 4.5a	3, 47	1.05	0.380
At leaf	53.3 ± 5.2a	53.1 ± 5.7a	55.8 ± 5.3a	60.6 ± 6.3a	3, 47	0.40	0.753
Silked off	0.7 ± 0.5a	0.7 ± 0.4a	2.9 ± 1.6a	0.8 ± 0.5a	3, 47	1.59	0.204
Unaccounted	11.0 ± 1.6a	11.6 ± 1.5a	15.3 ± 2.6a	13.3 ± 2.3a	3, 47	0.88	0.457

Means in row followed by the same letter are not significantly different (Fisher LSD, $P \leq 0.05$).

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Table 5. **Mean percentage of larvae recorded for the bioassay on influence of wind on silking behavior during the first four hours after eclosion for each behavior outcome for non-Bt near isoline with wind, non-Bt near isoline with no wind, MON810 with wind, and MON810 with no wind.**

Outcome	Mean ± SEM				ANOVA Statistical parameter		
	non-Bt isoline - Wind (n = 11)	non-Bt isoline - No Wind (n = 11)	MON810 - Wind (n = 11)	MON810 - No Wind (n = 11)	df	*F*	*P*
At leaf axil	50.8 ± 5.6a	55 ± 6.6a	39.5 ± 3.8a	44.3 ± 4.2a	3, 43	1.79	0.165
At leaf	31.3 ± 5.9a	31.9 ± 5.2a	35.8 ± 3.3a	43.2 ± 5.8a	3, 43	1.13	0.347
Silked off	3.7 ± 1.5b	0.5 ± 0.5b	15.1 ± 3.4a	2.1 ± 1.0b	3, 43	11.64	≤0.001
Unaccounted	14.2 ± 1.6a	12.5 ± 2.5a	9.1 ± 2.5a	10.5 ± 2.1a	3, 43	1.05	0.381

Means in row followed by the same letter are not significantly different (Fisher LSD, $P \leq 0.05$).

Table 6. Mean percentage of larvae recorded for the bioassay on neonate feeding for each behavior outcome at 6 h, 12 h, 24 h, and 48 h for non-Bt near isoline and MON810.

Outcome	Mean ± SEM		ANOVA Statistical parameter		
	non-Bt isoline (n = 10)	MON810 (n = 10)	df	*F*	*P*
6 hrs.					
found on leaf	37.4 ± 8.5a	30.5 ± 6.2a	1, 19	0.36	0.555
found in whorl	4.5 ± 1.7a	5.1 ± 2.2a	1, 19	0.03	0.858
found on bag	52 ± 8.1a	55.1 ± 6.2a	1, 19	0.15	0.705
unaccounted	6.1 ± 2.0a	9.3 ± 1.6a	1, 19	1.70	0.209
fed found on leaf	7.1 ± 3.2a	0.4 ± 0.4b	1, 19	4.53	0.047
fed found in whorl	0.0a	0.0a	1, 19		
fed found on bag	1.5 ± 0.8a	1.8 ± 1.0a	1, 19	0.07	0.796
12 hrs.					
found on leaf	35.5 ± 3.4a	19.2 ± 3.9b	1, 19	9.60	0.006
found in whorl	3.3 ± 1.0a	4.7 ± 3.0a	1, 19	0.17	0.689
found on bag	51.3 ± 5.2b	65.0 ± 3.5a	1, 19	5.05	0.037
unaccounted	10.0 ± 3.9a	11.1 ± 3.2a	1, 19	0.07	0.792
fed found on leaf	18.9 ± 2.9a	3.3 ± 1.5b	1, 19	22.31	≤0.001
fed found in whorl	1.5 ± 1.0a	0.0a	1, 19	2.25	0.151
fed found on bag	7.5 ± 1.5a	6.0 ± 2.4a	1, 19	0.34	0.566
24 hrs.					
found on leaf	56.2 ± 4.9a	20.8 ± 3.0b	1, 19	41.37	≤0.001
found in whorl	7.1 ± 2.1a	2.8 ± 1.0a	1, 19	3.29	0.086
found on bag	30.9 ± 4.0b	66.0 ± 3.2a	1, 19	43.29	≤0.001
unaccounted	5.9 ± 1.3a	10.4 ± 2.1a	1, 19	3.32	0.085
fed found on leaf	45.4 ± 5.5a	11.7 ± 1.9b	1, 19	42.42	≤0.001
fed found in whorl	5.8 ± 2.3a	1.0 ± 0.5a	1, 19	4.12	0.058
fed found on bag	18.5 ± 2.7a	24.4 ± 4.9a	1, 19	0.88	0.360
48 hrs.					
found on leaf	48.2 ± 4.2a	27.4 ± 2.7b	1, 19	17.36	0.001
found in whorl	12.2 ± 3.2a	0.8 ± 0.8b	1, 19	12.27	0.003
found on bag	23.1 ± 4.0b	49.9 ± 3.8a	1, 19	23.04	≤0.001
unaccounted	16.5 ± 3.3a	22.0 ± 4.4a	1, 19	1.01	0.329
fed found on leaf	36.3 ± 2.1a	16.5 ± 2.3b	1, 19	36.43	≤0.001
fed found in whorl	11.4 ± 2.9a	0.0b	1, 19	15.91	0.001
fed found on bag	12.7 ± 2.3b	26.6 ± 4.0a	1, 19	9.34	0.007

Log (x +1) transformations were performed to normalize the data. Means in row followed by the same letter are not significantly different (one-way ANOVA, $P \leq 0.05$).
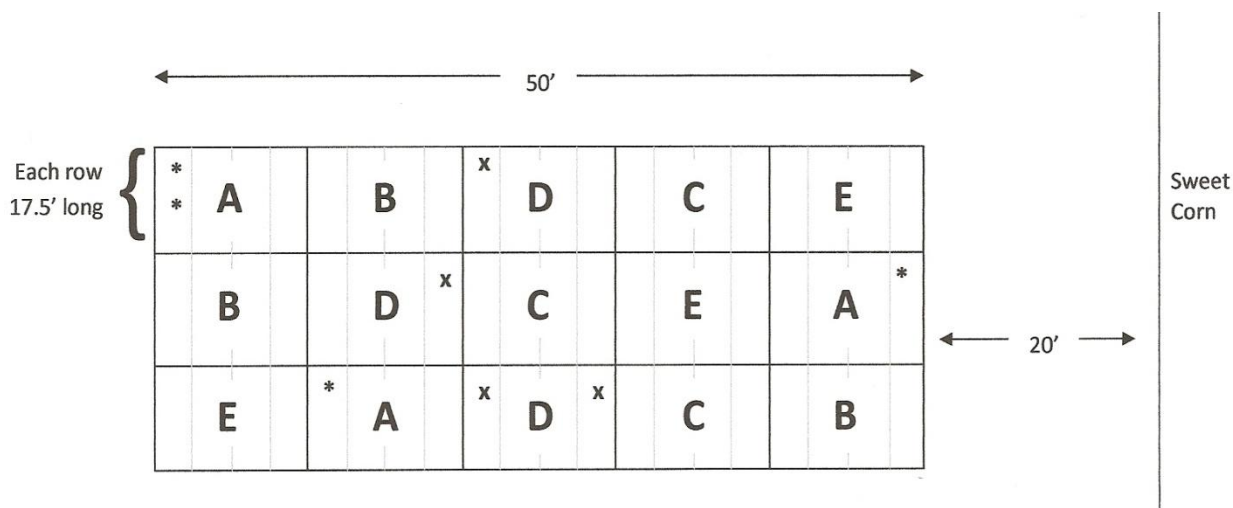
Figure 1. Pioneer Hi-Bred research plot evaluating the efficacy of a blended refuge against ECB. Plant abandonment field bioassay was established within the research plot for July 2009. Treatments used were (A) DAS 59122-7 × TC1507 × MON810 × NK603 stacked pyramid Bt corn and (D) non-Bt near isoline. 4 plants were randomly assigned for pyramid (indicated by *) and non-Bt near isoline (indicated by x). The sweet corn field was approximately 20' from the research plot.
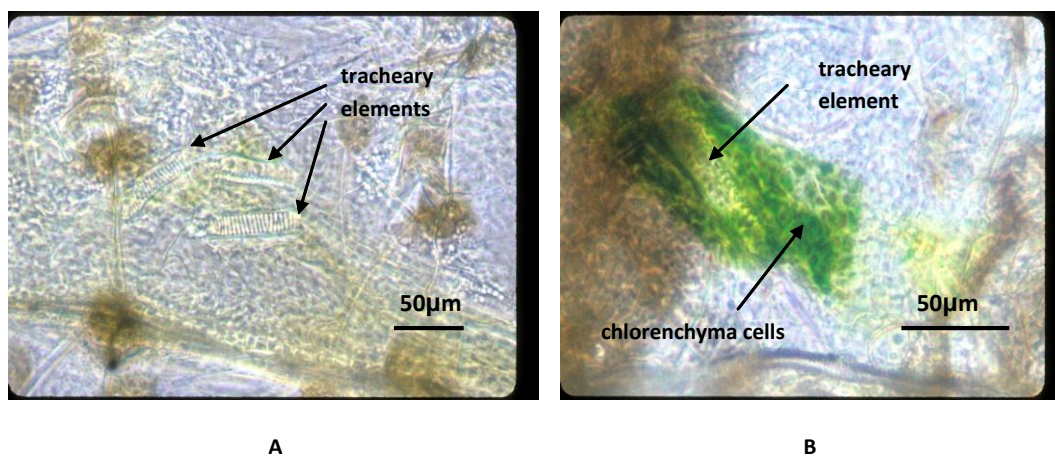
Figure 2. **Plant tissue inside the gut of neonate ECB. A) Tracheary elements in the gut of a neonate that had fed on non-Bt near isoline for 48 h.; B) Tracheary elements and chlorenchyma in the gut of a neonate that had fed on MON810 for 48 h. (Photos by T. Pizzolato).**
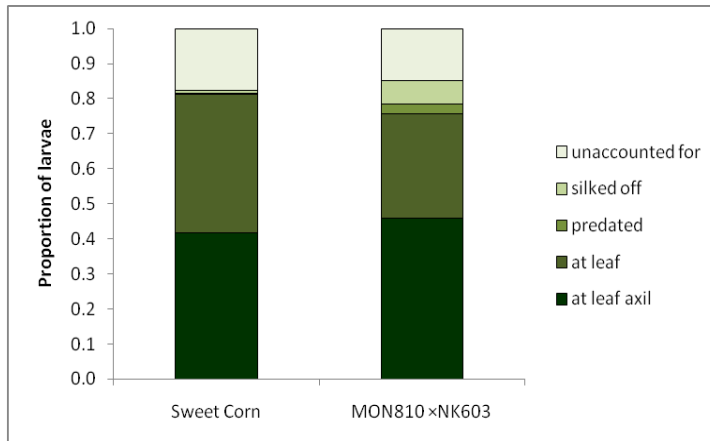
Figure 3. Mean proportion of larvae recorded for each behavioral response for the preliminary study conducted in the field during the first hour after eclosion for sweet corn and pyramid (MON810 × NK603) Bt corn.
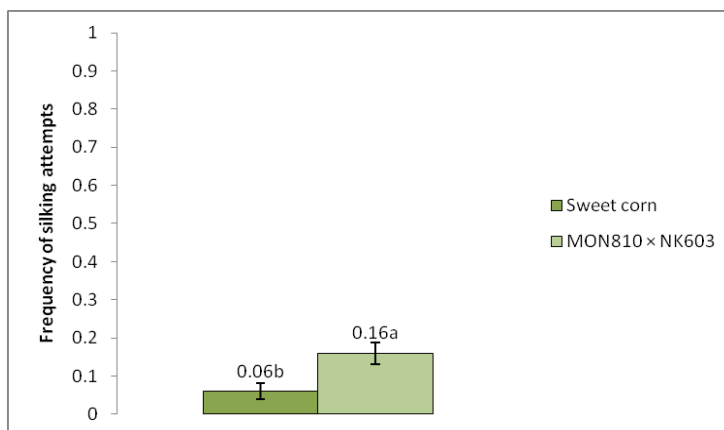
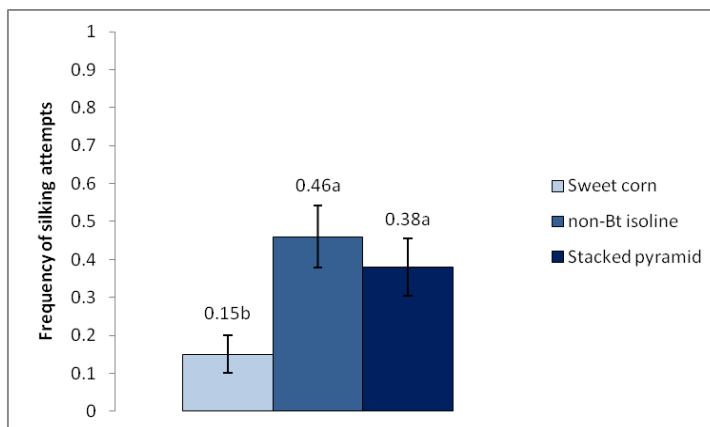**Figure 4.** Adjusted mean frequency of silking attempts (# of silking attempts/total # of larvae hatched) for the preliminary study in the field during the first hour after eclosion for sweet corn and pyramid (MON810 × NK603) Bt corn. Means followed by the same letter are not significant (Fisher LSD, $P \leq 0.05$).
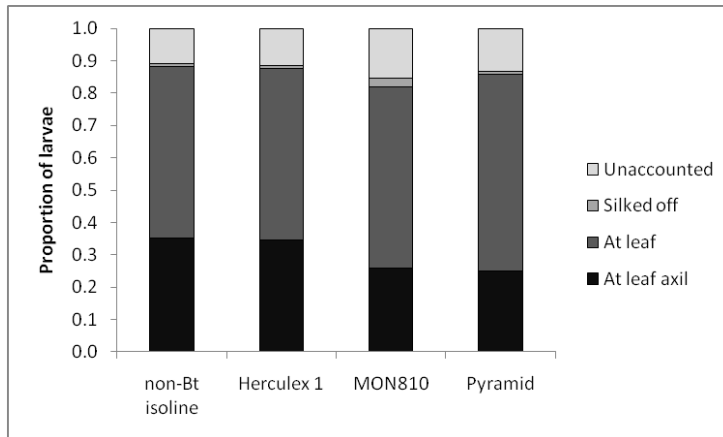
53
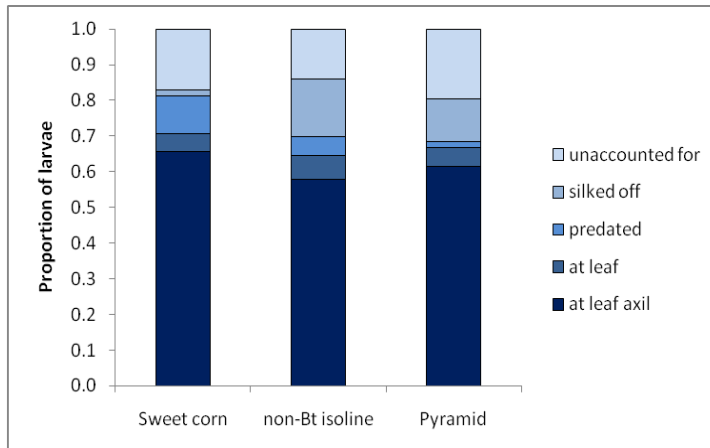
Figure 5.   **Mean proportion of larvae recorded for each behavioral response for the plant abandonment bioassay in the field during the first four hours after eclosion for sweet corn, non-Bt near isoline, and stacked pyramid (DAS 59122-7 × TC1507 × MON810) Bt corn.**
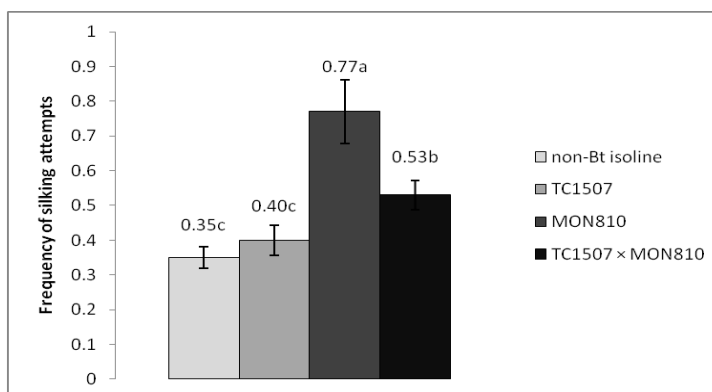

Figure 6.   **Adjusted mean frequency of silking attempts (# of silking attempts/total # of larvae hatched) for the plant abandonment bioassay in the field during the first four hours after eclosion for sweet corn, non-Bt near isoline, and stacked pyramid (DAS 59122-7 × TC1507 × MON810) Bt corn. Means followed by the same letter are not significant (Fisher LSD, $P \le 0.05$).**

Figure 7. Mean proportion of larvae recorded for each behavioral response for the plant abandonment bioassay in the laboratory during the first four hours after eclosion for non-Bt near isoline, TC1507, MON810, and pyramid (TC1507 × MON810) Bt corn.
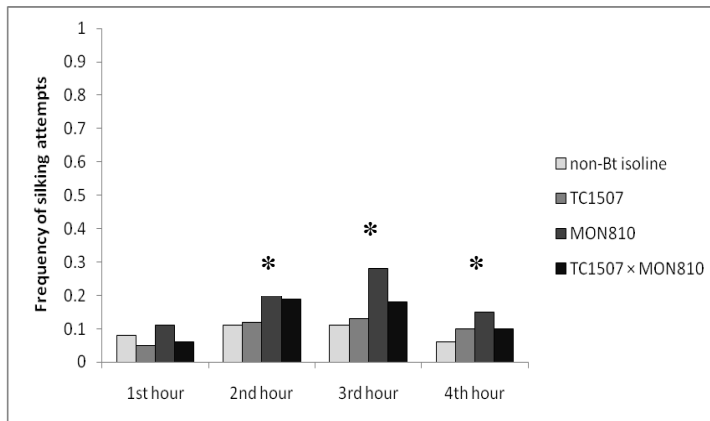

Figure 8. Adjusted mean frequency of silking attempts (# of silking attempts/total # of larvae hatched) for the plant abandonment bioassay in the laboratory during the first four hours after eclosion for non-Bt near isoline, TC1507, MON810, and pyramid (TC1507 × MON810) Bt corn. Means followed by the same letter are not significant (ANOVA and Fisher LSD, $P \le 0.05$).

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Figure 9. Adjusted mean frequency of silking attempts (# of silking attempts/total # of larvae hatched) for the plant abandonment bioassay in the laboratory categorized by hour for the first four hours after eclosion for non-Bt near isoline, TC1507, MON810, and pyramid (TC1507 × MON810) Bt corn. * indicate significance between treatments (Fisher LSD, $P \leq 0.05$).
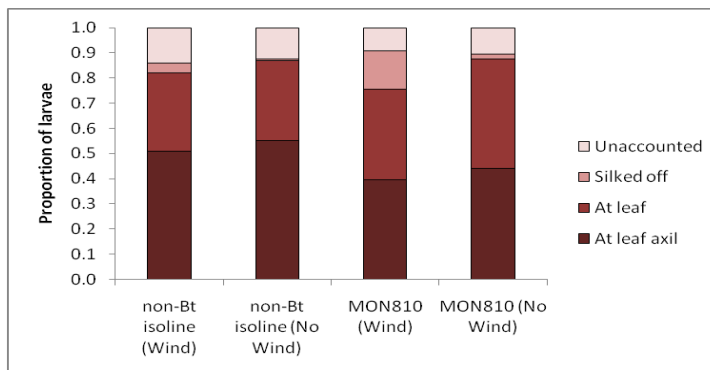

Figure 10. Mean proportion of larvae recorded for each behavioral response for the bioassay on the influence of wind on silking behavior during the first four hours after eclosion for non-Bt isoline with wind, non-Bt isoline with no wind, MON810 with wind, and MON810 with no wind.
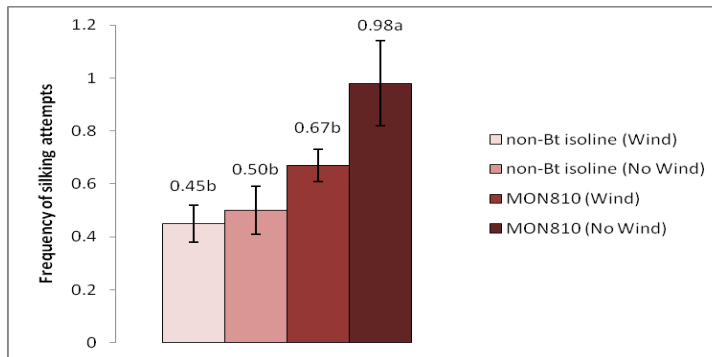
Figure 11. Adjusted mean frequency of silking attempts (# of silking attempts/total # of larvae hatched) for the bioassay on the influence of wind on silking behavior during the first four hours after eclosion for non-Bt isoline with wind, non-Bt isoline with no wind, MON810 with wind, and MON810 with no wind. Means followed by the same letter are not significant (Fisher LSD, $P \leq 0.05$).
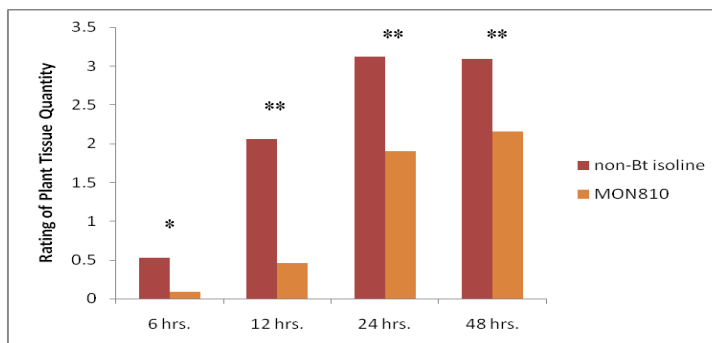

Figure 12. Mean plant tissue quantity rating values for larvae in the bioassay on neonate feeding that fed that were found on the leaf on non-Bt near isoline and MON810 at 6 h, 12 h, 24 h, and 48 h. Significant differences between non-Bt near isoline and MON810 are indicated by * for $p \leq 0.01$ and ** for $p \leq 0.001$ (one-way ANOVA, $P \leq 0.05$).

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