Resistance management of the western corn rootworm (*Diabrotica virgifera virgifera*):

behavior, survival and the potential for cross resistance on Bt corn in the field,

greenhouse and laboratory

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A Dissertation

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Doctorate of Plant, Insect & Microbial Science

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By

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The undersigned, appointed by the dean of the Graduate School,

have examined the Dissertation entitled

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VIRGIFERA VIRGIFERA): BEHAVIOR, SURVIVAL AND THE POTENTIAL FOR CROSS

RESISTANCE ON BT CORN IN THE FIELD, GREENHOUSE AND LABORATORY

Presented by Sarah N. Zukoff

A candidate for the degree of

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And hereby certify that, in their opinion, it is worthy of acceptance.

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ABSTRACT

The Environmental Protection Agency recently registered seed blend refuges for two of the transgenic Bt corn products targeting the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte. Larval movement between Bt and isoline plants can be detrimental to resistance management for high dose Bt products because the insect larvae will potentially be exposed to sublethal amounts of the Bt, however, the effect of this movement on low to moderate dose products is unknown. All current rootworm products are low dose. The main criteria for whether movement by WCR larvae between isoline and Bt corn plants will influence the development of resistance is whether or not selection for resistance is taking place. We found that movement between isoline and SmartStax® hybrid plants did occur in seed blend scenarios in our field study. The majority of plant damage to the SmartStax plants occurred when the larvae moved from surrounding infested isoline plants moved late in their development. These older, larger larvae are all able to tolerate the Bt in the plants, therefore resistance will likely not develop in these larvae. In a similar experiment, movement also occurred between Agrisure® Duracade™ and isoline plants in seed blend scenarios, however the damage was low for all treatments. With isoline plants being mixed with Bt plants in seed blend refuges, host recognition behavior of the western corn rootworm on Bt and isoline plants is also important to understand. There were no differences between the host recognition behavior of WCR larvae after exposure to mCry3A, Cry3Bb1, Cry34/35Ab1, or their isoline corn hybrids, therefore all hybrids were

perceived as hosts by WCR larvae. With all the hybrids on the currently registered being pyramided by different companies to control rootworms, the potential for cross resistance between these hybrids was evaluated using field resistant and susceptible populations. Based on the data from laboratory and greenhouse assays, the potential for cross resistance between mCry3A and Cry3Bb1 might be likely, but not between these hybrids and Cry34/35Ab1. Information gathered in this study provides important behavioral information on western corn rootworms that will aid in making decisions involving Bt corn hybrids.

CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

Introduction

There are over 95 million acres of corn, *Zea mays* L., planted in the U.S. with yields steadily increasing over the past 70 years due to improved breeding, technology, and production practices (USDA 2011). The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is considered to be corn's most important insect pest in the U.S. (Stamm et al. 1985, Krysan and Miller 1986, Gray et al. 2009). Crop losses and control costs due to rootworms is estimated to be between $1-2 billion annually in the U.S. and potential costs in Europe are estimated at €0.472 billion (Metcalf 1986, USDA-ARS 2001, Rice 2004, Mitchell 2011).

WCR larvae are subterranean and feed almost exclusively on the roots of corn (*Zea mays* L.), however they have also been shown to survive on a limited number of other species in the Family Poaceae (Branson and Ortman 1970, Clark and Hibbard 2004, Oyediran et al. 2004). WCR have a univoltine life cycle with three larval instars. Eggs of the WCR are laid in the soil in July-September and overwinter underground, hatching the following late May-early June. The eggs are often oviposited into cracks in the soil (Kirk 1979) or earthworm burrows (Kirk 1981) near the plant base, up to 15 cm under the soil surface. The adults feed primarily on silks, pollen and leaves of the corn plant.

The larvae are the most damaging stage of the WCR due to intense feeding on the root systems which causes the plant to have difficulty with nutrient and water uptake (Apple and Patel 1963, Kahler et al. 1985, Sutter et al. 1990). WCR larval damage also causes changes in photosynthetic rates, but stomatal function does not appear to be interrupted (Godfrey et al. 1993). This feeding damage does reduce grain yield, however (Godfrey et al. 1993). Later instars of the larvae move to the plant base and attack new root whorls as the plant grows (Strnad and Bergman 1987b). When severe damage occurs, entire nodes may be completely missing which predisposes the maize to lodging. Lodging occurs when the plant base is weak and the plant tips over, usually during heavy precipitation or high winds, causing further reductions in yield because lodged plants are more difficult to pick up with mechanical harvesters. Injury to the corn roots is measured by the 0-3 Node Injury Scale (NIS), where zero is no damage, and three nodes eaten to within 4 cm of the stalk gets the highest rating possible (Oleson et al. 2005).

An understanding of the biology of WCR and its interactions with the environment is important for making informed management decisions, creating new management tactics, and preventing resistance to current management practices. Biological information, such as host location, feeding preferences, and larval movement, is used to manage populations through an integrated approach of using multiple tactics to minimize damage to corn.

Host Location

The larvae of WCR are subject to a multitude of factors in their soil microenvironment that can affect their search for a host plant. Movement through soil is affected by soil porosity, soil type, moisture, and bulk density (Krysan 1999). The moisture of the soil can have an effect on larval survival, with inadequate moisture having a desiccating effect on larvae (Turpin and Peters 1971). Soil too saturated with moisture prevents larvae from moving through pores in the soil (MacDonald and Ellis 1990). When CO_2 was put into an arena an attractant with different soil types (sandy loam, silt loam and sand), larval movement was not inhibited, and larvae were recovered from close to the CO_2 source regardless of soil type (Strnad and Bergman 1987a). Pore space in soil becomes smaller at increasing bulk densities and in laboratory experiments, neonate larvae have been shown to travel less than 5 cm in compacted soils of sandy loam, silt loam and sand (Strnad and Bergman 1987a). First instar larvae were recovered from test arenas 30.5 cm long after 6 hours with non-compacted soil (Strnad and Bergman 1987a). During the duration of their lifespan, larvae were found to travel up to 100 cm in field studies conducted by Suttle et al. (1967), however, in a later study by Short and Luedtke (1970), no larval migration occurred after distances of 80 cm.

When first instar WCR larvae leave the egg and begin their search for host roots, they use specific chemical signals in their soil environment, such as CO_2 emitted from plant roots, to guide them toward potential hosts. In the absence of signals from nearby

potential hosts, WCR larvae will exhibit a "ranging" behavior, where they move quickly

and cover a wide area (Strnad and Dunn 1990, Bell 1991). In bioassays, after a 5 min

contact with and removal from a non-host plant, WCR larvae continue to exhibit ranging

behavior, but after 5 min contact with and removal from a host they exhibit a localized

movement behavior (Strnad and Dunn 1990). Roots of all plants give off CO_2 and many

soil insects are attracted to CO_2 (Jones and Coaker 1977, Strnad et al. 1986, Nicolas and

Sillans 1989, Bernklau and Bjostad 1998). WCR larvae are also highly attracted to CO_2

and likely use it to narrow their search for host roots (Strnad et al. 1986). If neonate

WCR larvae are removed from a host, they initialize a "localized search", which involves

a more restricted area of search with greater number of turns and a decrease in speed

of the larvae (Hibbard and Bjostad 1988, Strnad and Dunn 1990, Bell 1991). Localized

search keeps larvae within food, while ranging behavior is used to locate food patches

(Strnad and Dunn 1990).

At one time, CO_2 was thought to be the only long range attractant to WCR larvae

(Bernklau and Bjostad 1998), but (*E*)-β-caryophyllene was also found to be attractive

(Robert et al. 2012b, 2012a) and Robert et al. (2012c) suggested that WCR larvae use

hydroxamic acids as foraging cues. Bjostad and Hibbard (1992) had documented this

earlier. Hibbard et al. (1994) also suggested that long-chain free fatty acids were

involved in host location, while Bernklau and Bjostad (2008) documented these

compounds to be part of a feeding stimulant blend for neonate WCR larvae. Carbon

dioxide is the most potent attractant and will direct larvae to roots, but larvae use

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additional compounds to make actual host choices (Hibbard and Bjostad 1988).

Bernklau et al. (2009) used bioassays (Strnad and Dunn 1990) to isolate compounds that elicit localized search behavior, and they found that these compounds have "little or low volatility". They also determined that the behavioral responses of larval WCR depend on the type of compounds present, much more than the quantity of these compounds. The duration of localized search depends on the intensity or size of the host signal, and if the stimulus is removed, larvae eventually give up localized search and initiate a "ranging" behavior (Nakamuta 1985, Strnad and Dunn 1990). This behavior switching was found to be gradual. Strnad and Dunn (1990) observed that four hours after larvae had been removed from maize roots, they still had not covered the same amount of area searched or the velocity of a control which had never been exposed to host roots. Non-diapausing WCR larvae may exhibit slightly different behavior than diapausing larvae, in that non-diapausing larvae will initiate localized searching more often (Prischmann et al. 2009), but differences in damage to corn in the field are minimal (Hibbard et al. 1999b).

Once WCR larvae find a potential hosts root, contact cues are picked up by the maxillary palps to aid in feeding decisions (Branson and Ortman 1969). The compounds encountered will act either as a phagostimulant, such as sugars, or they will be deterrents, such as phenolic compounds (Johnson and Gregory 2006). Phagostimulants used by the rootworm larvae have been identified as a blend of short chain sugars and long chain fatty acids (Bernklau and Bjostad 2008). This blend is a combination of simple sugars, 30:4:4 mg/ml glucose:fructose:sucrose, and one of the free fatty acids in

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germinating corn roots, either oleic acid or linoleic acid (Bernklau and Bjostad 2008).

Interestingly, individual components by themselves did not elicit a major feeding

response by WCR larvae, but together, they did (Bernklau and Bjostad 2008). Recently,

Robert et al. (2012b) discovered that WCR larvae are attracted (E)-β-caryophyllene

which is an induced plant volatile given off when WCR larvae are feeding on certain corn

varieties. Roberts et al. (2012a) reported that larvae do best when they feed in a group

of 3-9 larvae on the plant size evaluated, but that they tend to have decreased

performance if there are 12 larvae present on a host plant. WCR can actually detect the

amount (E)-β-caryophyllene being given off by the host plant and can make host choices

based on the concentration of the volatiles present (Robert et al. 2012a). Robert et al.

(2012a) suggest that larvae have the ability to choose the plant with the perfect number

of other larvae present on the host for optimal feeding and performance when given a

choice. Interestingly, WCR larvae also use ethylene to locate host roots, whose

production is turned off when an above ground herbivore feeds on the host (Robert et

al. 2012b). This in turn, deters larvae from host roots and results in poor performance if

fed upon (Robert et al. 2012b). In contrast, when conspecifics have previously fed on

roots and (E)-β-caryophyllene is induced, WCR have increased performance when they

later encounter the root (Robert et al. 2012b).

Additionally, hydroxamic acids may play an important role in feeding and host

recognition, and levels of these compounds can vary depending on the maize line

evaluated as well as geographic location (Xie et al. 1992a). Bjostad and Hibbard (1992)

isolated and identified 6-methoxy-2-benzoxazolinone (MBOA) as the most attractive

component from crude corn extracts to the WCR larvae when equal levels of carbon

dioxide were on both sides of the choice. Although this compound is a chemical defense

against other insects, such as the European corn borer (Klun et al. 1967, Reed et al.

1972), it is not toxic to the WCR larvae (Abou-Fakhr et al. 1994), and is, in fact, used by

the WCR larvae to distinguish host plants. This compound is found almost exclusively in

maize and other grasses and rarely in non-host plants (Bjostad and Hibbard 1992). In

contrast to MBOA, DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)

concentrations have been shown to be correlated to WCR performance, but levels

higher than >1,000 µg/g fresh weight are needed to actually inhibit development (Xie et

al. 1992a). Even at high DIMBOA levels, some larval development actually occurred (Xie

et al. 1992a). DIMBOA levels decline over time in maize roots (Xie 1991), however lines

of corn that produce extremely high levels have been correlated with production of

inferior adults (Xie et al. 1990). Robert et al. (2012c) found that WCR larvae are resistant

to DIMBOA, and that rootworm larvae will use this compound to find the most

nutritious crown roots. However for the closely related generalist *Diabrotica balteata*,

DIMBOA was a deterrent. High DIMBOA lines did not have reduced WCR damage in

Missouri (Bruce Hibbard, unpublished data).

    Although neonate larvae can survive starvation for up to 96 hours, larvae need

to locate a host root within 12-36 hours or risk being too weak to burrow into the root

(Strnad and Bergman 1987a). Older larvae can survive food deprivation for up to eight

days depending on temperature, however the majority will survive up to three days at any temperature (Branson 1989, Oloumi-Sadeghi and Levine 1989). Clark et al. (2006) found similar results. Olmer and Hibbard (2008) found that second instar larvae can survive at least 5 days of starvation.

Feeding behavior within root

Larvae may distinguish not only hosts from non-hosts, but also different parts of host roots by tasting the different blends of compounds with their mouthparts (Johnson and Gregory 2006). Once a suitable host plant or host portion is found, WCR larvae will usually not stay in one area of the root, but move throughout the host roots in search of newer, younger roots on which they prefer to feed as the plant grows (Strnad and Bergman 1987b). As WCR larvae are not capable of completing their development on older roots (Hibbard et al. 2008b), these younger root whorls may not only be preferred, (Strnad and Bergman 1987b) but also required (Hibbard et al. 2008b). Strnad and Bergman (1987b) observed that a greater number of larvae preferred to feed on the distal portion of the roots. They postulated that more CO_2 is produced at the growing tip of the roots, which the larvae will then follow to the source and enter near this distal portion. Clark et al. (2006) found that over time larvae feeding on isoline corn would move from the tip area where cell formation occurs to the elongated portions above this area. Robert et al. (2012c) found that nutrient rich crown roots emit DIMBOA in higher amounts than other parts of the roots, and larvae grew best on these roots. First instar WCR larvae were found to feed in seminal roots as well as in root whorls 1-7 (one

being the oldest and 7 the youngest) (Strnad and Bergman 1987b). They preferred

maize roots that were 2.0 mm or less in diameter with the smallest roots that larvae

where observed to enter being only 0.5 mm in diameter (Strnad and Bergman 1987b).

They observed second instars feeding on root whorls 1-8 and third instars feeding on

root whorls 1-9. Third instars were never observed to feed on seminal roots (Strnad and

Bergman 1987b). In contrast, larvae were never found in root whorls 1-2 in assays done

with northern corn rootworm, *Diabrotica barberi*, by Apple and Patel (1963), and they

hypothesized that these roots were too lignified for larvae to feed on. Older roots have

higher levels of lignin (Zeier et al. 1999) which may be difficult for larvae to ingest and

may be used as a defense mechanisms in maize (Campbell 1996). In Strnad and

Bergman (1987b), the corn was younger than corn used by Apple and Patel (1963), so

roots in whorls 1-2 may have been softer and more palatable to larvae.

Riedell and Kim (1990) observed that WCR larvae feed in the cortex tissue within

roots, avoiding the pith area and vascular system. Both of these root tissues were

considered to be identical in nutrition, however, the cell walls may have prevented

larvae from entering vascular tissue (Riedell and Kim 1990). Riedell and Kim (1990)

found that unless cut roots were sealed on the end in paraffin larvae would readily feed

on the pith area. In maize, cell walls become lignified as roots grow (Peterson et al.

1982). This was thought to discourage larvae from feeding on pith and vascular tissues

(Riedell and Kim 1990) as well as older root tissues (root whorls 1 and 2) in older corn.

Western corn rootworm larvae are capable of behavioral plasticity when making feeding decisions. Larvae may have the ability to detect and avoid areas of certain compounds in corn roots that might be detrimental to them. For example, in behavioral bioassays, WCR larvae bored into untreated control roots more than roots treated with varying hydroxamic acids (Xie et al. 1992b). The ability of WCR larvae to not only discern compounds on or within the root, but to also modify their behavior based on these stimuli, has the potential to lead to behavioral resistance.

Movement between plants

WCR larvae not only move within plant roots, but they have also been shown to move between plants after initial establishment. Many factors can influence larval movement between plants such as food availability, compounds present in the maize, toxic proteins found in transgenic corn, as well as whether or not potential hosts are nearby. Plant to plant movement by WCR appears to be primarily driven by food availability. Density-dependent factors (i.e. competition) for food, will affect larval movement. If high amounts of damage occur to a host plant by conspecifics, larvae must leave to find additional food sources in order to survive. Hibbard et al. (2004) demonstrated that larvae move to a neighboring plant only after a significant (>0.75 NIS) amount of damage has occurred. When high levels of WCR eggs were artificially infested, larvae moved only after high amounts of damage occurred, not during initial establishment (Hibbard et al. 2004). Hibbard et al. (2003) found that after initial

establishment on plant roots, WCR larvae can move at least three plants down a row (~0.45 meters) as well as across a 0.46 meter row.

WCR larval movement between Bt and isoline maize may be facilitated by a feeding preference for isoline plants. In laboratory experiments, larval recovery was not significantly different between isoline and Bt (Cry3Bb1) treatments, however greater numbers of larvae fed on isoline corn rather than Bt roots (Clark et al. 2006). These larvae exhibited non-preference behavior for Bt (Cry3Bb1) roots (Clark et al. 2006). Clark et al (2006) also found that on isoline, larvae aggregated at the tip, only later moving to older tissue. In contrast to this, on Bt (Cry3Bb1) larvae exhibited a ranging behavior, stopping to sample root hairs or small amounts of tissue, but were not observed actively feeding (Clark et al. 2006). Larvae on Bt (Cry3Bb1) plants that had ingested roots ceased movement while larvae exhibiting ranging behavior had visibly empty guts (Clark et al. 2006). Data from Hibbard et al. (2005) implies that larvae prefer isoline roots when given a choice between Bt (Cry3Bb1) and isoline plants in field experiments. Hibbard et al. (2005) also demonstrated that even though WCR larvae seem to prefer isoline roots larvae will move from a highly damaged isoline plant to adjacent Bt plant in search of food.

Insect Resistance Management

Companies that produce transgenic maize plants that contain Bt proteins are mandated by the EPA to develop insect resistance management plans (IRM). These IRM plans contain requirements to plant non-Bt refuge plants which produce susceptible

insects. These will be available to mate with resistant insects that emerge from Bt plants thereby delaying resistance to Bt, assuming resistance is recessive. The use of Bt hybrid corn carries many benefits to human health and the environment by reducing the need for synthetic insecticides. Maintaining continued susceptibility to Bt prompted the adoption of these IRM requirements (EPA 1998). The original refuge strategies assumed that Bt titer is high, resistant individuals are rare, and resistant genes are recessive (EPA 1998, Gould 1998, Tabashnik and Gould 2012), however all current Bt proteins rootworms are low to moderate dose (EPA 2003, Siegfried et al. 2005).

Larval susceptibility to Cry3Bb1 toxin amongst wild and lab populations of western corn rootworm for LC_{50} ranged from 2.01 µg/cm2 to 13.04 $µg/cm^2$ (Siegfried et al. 2005). In contrast, the LC_{50} for corn earworm (*Helicoverpa zea* Boddie) on Cry1A were 70.3 ng/cm^2 to 221.30 ng/cm^2 (Siegfried et al. 2000). Cry1A is considered relatively low dose against corn earworm when compared to European corn borer, yet the dose required to kill half a susceptible population is several orders of magnitude lower than what is required for WCR. This means that a greater proportion of rootworms are likely to survive, unlike the high-dose products that target above ground lepidopterans where surviving individuals are extremely rare (Tabashnik and Gould 2012). The EPA initially required a 20% refuge with most Bt lines that contain a single rootworm targeted gene when planted in the Corn Belt region (non-cotton growing region) of the US where rootworm threat is the highest, but this has since been reduced to 5% refuge for pyramided hybrids.

A newer refuge concept involves mixing Bt corn hybrid seed and their refuge

seed in the same bag for sale. These products are being called "Refuge in a bag" (RIB)

and referred to as "blended" or "mixed" refuges. This reduced and blended refuge

approach has arisen in response, in part, to noncompliance issues with planting of

refuges, which is thought to have contributed to field evolved resistance in Bt corn (Jaffe

2009, Gassmann et al. 2011). With rootworm targeted RIB style products being

approved for commercial sale by the EPA (EPA 2010 b,c, 2011a), a firm understanding of

resistance management of WCR larval movement between Bt and isoline plants is more

important than ever. Pioneer Optimum Acremax RW and Optimum Acremax 1 have a

10% blended refuge, while SmartStax corn (produced by a collaboration of

DowAgrosciences and Monsanto) has a 5% refuge (EPA 2011a). Syngenta's next-

generation rootworm product, Agrisure Duracade™, containing the eCry3.1Ab (event

5307) and mCry3a (event MIR604) rootworm-targeting toxins was recently deregulated

by USDA and is expected to be commercially available by 2014. This pyramided hybrid is

expected to be sold as a blended 5% RIB as well. Because resistance has evolved so

rapidly in WCR to Bt, Tabashnik and Gould (2012) argue that the minimum refuge for

single Bt hybrids targeting rootworms be raised to 50% and for multiple Bt genes 20% in

order to have a more sustainable future for rootworm management.

Control and Resistance: an evolving problem

There are a number of management tactics used to reduce WCR damage to corn

plants. The primary method used in most regions during the last 100 plus years is crop

rotation. Granular soil insecticides have traditionally been the most common method of rootworm management in situations where continuous corn was planted (Levine and Oloumi-Sadaghi 1991).

Current soil insecticides include Force® (tefluthrin), a broad spectrum granular insecticide, and Aztec® (tebupirimiphos plus cyfluthrin) which can be applied using Smartbox® technology. Smartbox limits user exposure by minimizing contact with the product. These products are generally applied in a T-band over the row or put in the furrow with seed. There have been no instances of WCR resistance to soil insecticides applied in a band thus far, probably due to the fact that some rootworms are able to survive outside the band where the granules are active (Van Rozen and Ester 2010). Soil insecticide proved a viable control option even under moderate to heavy WCR infestations that were resistant to foliar insecticides such as methyl parathion and carbaryl (Wright et al. 1999). All current Bt products and refuge plants are treated with insecticidal seed treatments, usually Poncho® (clothianidin) or Cruizer ® (thiamethoxam) which are used at high rates for rootworm control, but also kill secondary pests such as wireworms. These products protect the germinating seed for up to 10 weeks, however the consistency of seed treatments may be affected by soil moisture, planting time, and seed coating (Van Rozen and Ester 2010).

The most common cultural control method utilized is rotation of maize with a non-host plant, such as soybeans (*Glycine max* L.). This has been a widely practiced method for controlling WCR for many years. When soybeans are planted in alternating

years with corn in a field, it disrupts the life cycle of the rootworms, as larvae cannot

survive on soybean roots. Rotation-resistant WCR populations developed initially in East

Central Illinois and West Central Indiana (Onstad et al. 1999, Levine et al. 2002, Gray et

al. 2009, Knolhoff 2010, Curzi et al. 2012). Recently, Curzi et al. (2012) found higher

levels of cathepsin L in rotation-resistant populations of WCR beetles that fed on

soybeans, allowing them to circumvent the soybean defense, cysteine protease

inhibitors, long enough to lay their eggs in soybean fields. These WCR eggs overwinter in

the soybean field, and when corn is planted the following year, they hatch and start to

feed on the corn plant. WCR adults are not attracted to soybeans, however many years

of crop rotation have decreased the plant heterogeneity of the landscape leaving WCR

adults with a higher probability of encountering soybeans when leaving natal corn fields

(Levine and Oloumi-Sadeghi 1996, Levine et al. 2002, Spencer 2005, Curzi et al. 2012).

Mabry et al. (2004) determined that, although WCR cannot survive on soybeans alone,

they can survive for a period when feeding on both corn and soybeans.

Foliar insecticides are occasionally needed to limit silk clipping by adult western

corn rootworms to limit egg laying in corn following corn fields were rotation is not

being practiced by producers (Pruess et al. 1974). Foliar insecticides were used in large

parts of Nebraska for many years (Meinke et al. 1998). Timing of sprays is critical for

effective control (Gerber et al. 2005, Van Rozen and Ester 2010), and is determined by

scouting. Scouting involves counting the number of beetles per plant for threshold

determination, as well as monitoring rootworm adult emergence using sticky traps.

Methyl parathion and carbaryl were commonly used aerial insecticides (Chandler et al. 2008). A common aerially applied insecticide is Penncap-M® which is encapsulated methyl parathion. Populations in parts of Nebraska have become increasingly resistant to Penncap-M as well as carbaryl applications (Meinke et al. 1998). Parami et al. (2006) determined that the field evolved resistance to methyl parathion is not associated with significant fitness costs and is still present even after selection pressure was removed. After several years of assays with resistant and susceptible WCR larvae and adults, esterase-mediated resistance to methyl parathion was discovered (Miota et al. 1998, Wright et al. 2000, Zhou et al. 2002, Zhou 2003). It was further determined that resistance to methyl parathion can be linked with increased Group II esterase proteins, and that a 66-kDa protein could be used as a resistance associated biochemical marker in assays for resistance monitoring (Zhou 2004, Zhou et al. 2005).

Insecticidal baits were developed to kill adult western corn rootworms after feeding on a mixture of insecticide and cucurbitans (strong feeding stimulants) (Chandler 2003) and a sticky carrier. The products SLAM® (Microflo Co. and BASF Corp.) and Compel® (Ecogen, Inc.) were insecticidal baits developed in the 1990's that included carbaryl as the insecticide. At that time, the USDA initiated their Areawide Pest Management Program (APMP) whose short-term goal was to include 75% of row crop producers in the U.S. in an Integrated Pest Management program (USDA 1993, 1994, Chandler et al. 2008). These baits were widely tested across the Corn Belt (Chandler 2008). Eventually, SLAM and Compel were discontinued (1998) and two new products

took their place as adjuvant carriers added to the insecticide of choice. These products were Invite® and CideTrak® CRW. Interestingly, Zhu et al. (2001) found reduced carbaryl susceptibility after repeated use of SLAM® in parts of Kansas. As products containing Bt genes became commercially available, rootworm targeted Bt hybrid popularity grew quickly and the need for baits dwindled (Chandler 2008).

Transgenic corn plants that produce Bt protein toxins have been a widely adopted method of controlling WCR since the introduction of rootworm targeted Bt in 2003. The first Bt rootworm targeted product was Monsanto's Yieldgard® Rootworm corn hybrid expressing the Cry3Bb1 protein registered in 2003. Since then, there have been several other products incorporating Cry proteins including, MIR604 utilizing mCry3a, MON88017 utilizing Cry3Bb1, and DAS59122-7 utilizing the Cry34/35Ab1 proteins, as well as pyramided hybrids incorporating one or more of these traits. Over 50 million acres in the U.S. being planted with Bt rootworm targeted hybrids in 2011 (Marra et al. 2012). Corn expressing plant incorporated protectants, such as those that utilize *Bacillus thuringiensis* (Bt) proteins, are the newest forms of WCR control.

Corn hybrids that contain one or more above ground and below ground Bt traits targeting pests and/or insecticidal and herbicide tolerance are considered "stacked", and hybrids that contain more than one Bt that targets the same pest complex are considered "pyramids". Bt has been a preferred option over insecticides for human health reasons. There are no known mammalian health impacts (Siegal 2001). In addition, Bt corn offers little to no effect to non-target insects (Al-Deeb and Wilde 2003,

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Ahmad et al. 2005). The use of Bt has significantly reduced the need for insecticides (Kline 2000, Carpenter and Gianessi 2001, Phipps 2002) until recently, when some populations of WCR have evolved resistance to certain Bt products (Gassmann et al. 2011).

Resistance to all three Bt proteins currently on the market has been selected in laboratory reared colonies of WCR (Lefko et al. 2008, Meihls et al. 2008, Binning et al. 2009, Meihls 2011). In the laboratory, selection for resistance was accomplished after as little as three generations for Cry3Bb1 (Meihls et al. 2008). Selection pressure in the laboratory was accomplished by allowing only rootworms that survived to adulthood after being constantly exposed to Bt, to mate (Meihls et al. 2008). This extreme selection does not allow for immigration by susceptible insects which would slow evolution of resistance (Onstad et al. 2001). Unfortunately, field evolved resistance to Cry3Bb1 of WCR has been reported from Iowa (Gassmann et al. 2011, Gassmann 2012, Gassmann et al. 2012), Illinois, Minnesota, Nebraska, and South Dakota (Porter et al. 2012). These areas of Cry3Bb1 Bt failure have higher than expected damage to the roots (e.g. greater than 0.75 on the node injury scale), and almost all these fields have a history of corn after corn for multiple years (usually with Cry3Bb1 expressing corn).

Some of these failures have been attributed to improper planting of refuges (Jaffe 2009, Gassmann et al. 2011), thereby creating an intensive selection scenario in the field similar to what Meihls (2008) and others created under laboratory conditions. Currently, crop rotation with a non-host is being emphasized to combat Bt failure,

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rotating to pyramided rootworm targeted traits or using a non-Bt hybrid plus soil insecticides or ultimately, the judicious use of rescue sprays with insecticides. Despite failure of Cry3Bb1 in certain areas, Bt still remains a viable option for WCR larval control due to its minimal impacts on human and environmental health, however rootworm scientists, the EPA, and seed companies are currently rethinking and updating current resistance management plans to combat this resistance issue (EPA 2010a, 2011a, b).

Maize cultivars containing natural resistance to WCR are uncommon and not yet commercially available. Experimental maize lines which include plants with larger root systems that withstand higher levels of rootworm damage and still maintain yields are considered rootworm tolerant lines (Ortman et al. 1974, Branson et al. 1983, Branson and Sutter 1989, Xie et al. 1992b, Hibbard et al. 1999a, Hibbard et al. 2008a). Native resistance, via antibiosis, to WCR has been documented in at least two maize genotypes SUM2162 and SUM2068 (El Khishen et al. 2009, Bernklau et al. 2010).

Information related to resistance management of western corn rootworm is important for making science based management decisions and ensuring the long term use of Bt technology and all other available control options. This study focuses on using WCR larval behavior to understand and predict problems with Bt resistance before they arise. This manuscript will explore behaviorally mediated resistance to Bt hybrids; larval movement in newly adopted RIB style fields, and cross resistance within pyramided Bt products. This study combines laboratory, greenhouse and field experiments conducted at the University of Missouri/USDA-ARS facilities in Columbia, MO during 2009-2013.

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CHAPTER II: WESTERN CORN ROOTWORM LARVAL MOVEMENT IN SMARTSTAX SEED BLEND SCENARIOS

Introduction

Information on the movement potential of larvae of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is relatively well understood for a soil insect. Movement through the soil is affected by soil bulk density (Strnad and Bergman 1987a), soil moisture (Macdonald and Ellis 1990), and macropores in the soil (Gustin and Schumacher 1989). If a newly hatched western corn rootworm does not locate a suitable host within 24 hrs, its likelihood of surviving to the adult stage is significantly decreased (Branson 1989). Distance to host plants can also affect establishment. Plant damage and lodging were reduced when artificial infestation was farther (>22.5 cm) from the plant when compared with infestation closer (7.5 or 15 cm) to the plant (Chaddha 1990). Other factors also influence larval movement. For instance, western corn rootworm larvae are highly attracted to carbon dioxide (Strnad et al. 1986, Hibbard and Bjostad 1988) which is released from respiring roots (Massimino et al. 1980). Additional contact cues from roots trigger a localized search behavior when larvae are removed from the host and this localized search behavior is not triggered by non-host roots (Strnad and Dunn 1990, Bernklau et al. 2009).

Larval movement is not complete when the neonate reaches a suitable host plant. Strnad and Bergman (1987b) demonstrated that later instar larvae re-distribute, moving to younger root whorls that emerge from the stalk as the plant develops. Larval

feeding on these root whorls as they begin elongation from the stalk is responsible for their pruned appearance. The extent of western corn rootworm larval movement that occurs between plants and rows within a corn field after initial establishment was first evaluated by Hibbard et al. (2003). They found that larval movement from highly damaged, infested plants to nearby plants with little to no previous root damage occurred and that row spacing, but not plant spacing, significantly affected this movement. Hibbard et al. (2004) further evaluated the effect of egg density on establishment and post-establishment larval movement and damage to corn. Initial establishment on a corn plant was not density-dependent because a similar percentage of larvae were recovered from all infestation rates. Plant damage and, secondarily, subsequent larval movement were density-dependent. Very little damage and movement occurred at lower infestation rates, but significant damage and movement occurred at higher infestation rates. Movement generally occurred at a similar time as significant plant damage and not at initial establishment, so timing of movement appeared to be motivated by available food resources rather than crowding. Hibbard et al. (2005) evaluated larval movement in non-Bt corn plots, MON863 transgenic corn expressing the Cry3Bb1 protein, and two types of possible seed-blend scenarios. The number of western corn rootworm larvae recovered from MON863 plants adjacent to infested, isoline plants was low and not statistically significant either year. The study showed that both neonate and later instar western corn rootworm larvae preferred isoline roots to MON863 roots when a choice was possible. However, when damage to

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the infested isoline plant was high, western corn rootworm larvae apparently moved later in the season to neighboring MON863 plants and caused significant damage (Hibbard et al. 2005). Although extractable Cry3Bb1 decreased from V4 to V9 stage corn (Vaughn et al. 2005), no significant difference in activity against neonate western corn rootworm larvae was noted between V3 and VT stage corn (Ritchie et al. 1992) from MON863 (Hibbard et al. 2009) or mCry3a (Frank et al. 2011).

Meihls et al. (2008) evaluated the development of resistance to Cry3Bb1 (MON863) corn under full transgenic rearing (constant Bt exposure) and two types of seed-blend scenarios (early and late Bt exposure) in the lab/greenhouse. Full rearing on MON863 led to resistance within three generations. Selection for resistance when first instar larvae fed on isoline corn and third instars fed on MON863 (second instars fed on both) led to the development of resistance within six generations of selection (Meihls et al. 2008). The field resistance ratio for this colony was reduced to 3.6 instead of 11.7 for the colony reared fully on MON863 as larvae. The resistance ratio of the colony simulating movement of neonate larvae from MON863 to isoline corn was 0.3 indicating this colony was more susceptible to MON863 in no choice field experiments than the control colony (Meihls et al. 2008).

Gassmann et al. (2011) reported the first case of field evolved resistance of the western corn rootworm to Cry3Bb1 in Iowa. They found significantly higher survival of western corn rootworm larvae on Cry3Bb1 when from "problem" fields where farmers had reported severe root injury to Cry3Bb1 plants than when from control fields

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(Gassmann et al. 2011). Interestingly, western corn rootworm larvae from the problem fields did not survive on Cry34/35Ab1 any better than those larvae from control fields (Gassmann et al. 2011). The registration of a seed blend refuge for Pioneer's Optimum® AcreMax™ RW transgenic corn expressing the Cry34/35Ab1 protein (EPA 2010 b,c) and registration of seed blends for SmartStax™ seed by Monsanto Company and Dow AgroSciences (EPA 2011a) raise questions concerning larval movement and the potential for seed blends to affect the development of resistance. SmartStax transgenic corn expresses two rootworm traits including Cry34/35Ab1 and Cry3Bb1 proteins. These traits are expressed throughout the plant during all stages of corn development. Larval movement between Bt and isoline plants can be detrimental to high dose Bt products because the larger larvae are generally more tolerant of the Bt toxins and there is a greater potential for heterozygote individuals to survive. It is uncertain how larval movement between Bt and isoline plants will affect insect survival on moderate dose Bt toxins, but an understanding of larval movement is a first step. The purpose of this study was to evaluate western corn rootworm larval movement, damage, and survival in a SmartStax seed blend scenario.

Methods

The experiment was conducted at the Bradford Research and Extension Center 9 km east of Columbia, MO, USA. In both years, the field had been planted to soybeans (*Glycine max* L.) the previous year. We assumed the fields did not have a background western corn rootworm population because central Missouri does not yet have the

rotation-resistant population (Gray et al. 2009). The experiment was planted on May 15, 2010 and May 4, 2011. Hybrid seed was obtained from Monsanto Company and the same hybrid and seed lots (NB510 QQRA (SmartStax) and NB510 HTTZ (isoline seed), each with glyphosate tolerance, were used in both years.

The experimental unit for this study was a subplot consisting of 3 consecutive corn plants each spaced approximately 15 cm apart. The experimental design was a randomized complete block arranged in a split-split-plot (larval recovery and plant damage) or a split-plot design (beetle emergence) as outlined in Steel et al. (1997) in which the factors were arranged by a 8 by 5 by 2 for larval recovery (treatment×sample date×plant), 8 by 2 by 2 for plant damage (treatment×sample date×plant), and 8 by 2 for beetle emergence (treatment×plant). The eight treatments consisted of four combinations of SmartStax and isoline corn and two positions for rootworm egg infestations (at either the center or both end plants) each with five replications (Fig. 1) in 2010 and 2011. Each subplot had three consecutive plants that were either all SmartStax (Treatments 1, 5), all isoline (Treatments 2, 6), an isoline center plant surrounded by two SmartStax plants (Treatments 3, 7), or a SmartStax center plant surrounded by two isoline plants (Treatments 4, 8) (Fig. 1). Each three-plant subplot was either three plants in a straight row for Treatments 1, 2, 5 and 6, or a kernel of the opposite type (either SmartStax or isoline) was slightly offset from the row between plants for Treatments 3, 4, 7 and 8 (Fig. 1). Subplots in the same row were separated by at least 150 cm. All larval sampling and planting methods were modified from Hibbard

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et al. (2004). In 2010, each replication had 32 rows that were 15.2 m long and each of the eight treatments consisted of four of the 32 rows. In 2011, each replication had 16 rows that were 11.6 m long and each of the eight treatments consisted of two of the 16 rows. Each replication included subplots for plant damage (at two different evaluation times), adult emergence (two subplots needed because plant spacing did not allow sampling multiple plants within a subplot), and in 2010 only, five additional subplots were use to evaluate larval recovery at five different recovery times. The nine subplots used in 2010 and the four subplots used in 2011 within each treatment and replication were randomly assigned to sets of three plants with uniform plant spacing. Since Treatments 3, 4, 7, and 8 were planted at the same time as the other treatments, a total of 15 (2010) or 9 (2011) kernels of the opposite plant type were hand planted and marked with a stake just outside the row for each replication. The nine (2010) and the four (2011) subplots with the most uniform plant spacing were chosen from the 15 (2010) or 9 (2011) potential subplots. It was usually necessary to remove the middle plant within the original row at the time the subplots with the offset seed were chosen for Treatments 3, 4, 7, and 8. Plants in the original row were machine planted.

In order to assure each SmartStax plant expressed rootworm-targeted genes, gene check strips (EnviroLogix, Portland, ME) were utilized to verify that all 540 SmartStax plants used in the 2010 study expressed Cry3Bb1. In addition, a random sample of 45 SmartStax plants were also evaluated for Cry34Ab1. Finally, five isoline plants were also evaluated for Cry3Bb1 and Cry34Ab1. In 2011, all 240 SmartStax plants

were tested for the Cry3Bb1. All gene checks confirmed the presence of the targeted

genes for both years. For Treatments 1, 2, 3, and 4 the center plant of each subplot was

infested and for Treatments 5, 6, 7, and 8, the two end plants were infested (Fig. 1). The

location of infestation (Fig. 1) was chosen so that larvae were forced to move through

the infested plant prior to reaching any other plants in the subplot (with the possible

exception of Treatments 1 and 2). Approximately 1,700 western corn rootworm eggs

were used for each infested plant. Viability averaged 76.5%, so there were

approximately 1,300 viable eggs per plant infested in 2010. In 2011, viability averaged

77.5%, so viable eggs infested closely matched 2010. Natural western corn rootworm

egg infestations of 12.2×10^7 eggs per ha have been documented (Pierce and Gray 2006),

which is >2,800 eggs per 30.5 cm of maize row. The amount of damage that 1,300 viable

eggs typically cause is equivalent to a moderate infestation (Hibbard et al. 2004). Eggs

were placed ~10 cm deep and ~2.5 cm from the plant base. Plants were infested at ~V2-

3 (Ritchie et al. 1992) on June 8, 2010 and May 18, 2011. Wild type eggs were obtained

from French Ag. Research, Lamberton, MN and were originally from Dodge City, Kansas.

In 2010, wild type eggs (700,000) were augmented with 60,000 eggs from the primary

diapausing strain from the USDA-ARS laboratory in Brookings, SD to reach the target

number of eggs needed for the study. The Brookings strain causes similar damage in the

field to wild type strains (Hibbard et al. 1999b) and is genetically similar to field strains

(Kim et al. 2007). Subplots were infested as described in Fig. 1, except in 2010 the adult

emergence plots had only one of the two end plants infested (the north plant – row

direction was north/south) due to insufficient egg numbers and the size of emergence cages. In adult emergence subplots where the end plant was to be sampled, the north plant was always sampled in 2010. This difference in infestation locations between 2010 and 2011 is illustrated in Fig. 2. One additional difference between years was that on July 3, 2011, large hail destroyed most corn leaves and knocked over many plants resulting in premature death of many of the corn plants. Plants eventually grew out of the damage and appeared mostly normal later in the season.

Degree days were used to time sampling dates and began accumulating the day of infestation. They were calculated from the average 24 hour bare soil temperature at a depth of 5 cm and subtracting the developmental threshold of 11.1°C (Wilde 1971, Levine et al. 1992) for each day, though degree days could not be negative. Temperatures were obtained from the University of Missouri commercial agriculture automated weather station located at the Bradford Research and Extension Center, where the trials were conducted.

Larval Recovery

Larvae were sampled on five sample dates in 2010 with the first sample taken on June 8, after ~330 degree days, when approximately 90% egg hatch had occurred. Sampling dates then followed every 4 to 5 d. All three plants in each subplot were destructively sampled. Using techniques similar to Hibbard et al. (2004), the top of the plant was cut ~30 cm from the ground, the root ball and surrounding soil were extracted with the aid of a 4 pronged garden fork, and each root ball was carefully placed in a

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mesh bag in an attempt to keep the soil structure intact. The mesh bags containing

roots, soil, and larvae were hung in the greenhouse with the cooling system turned off

for approximately one week. Afternoon temperatures in the greenhouse averaged

40.4±0.6 °C from 13:00 to 16:00 h for all larval sample dates and the daily temperatures

(including evenings) averaged 28.51±0.40 °C, 30.13±0.54 °C, 31.68±0.48 °C, 33.24±0.64

°C, 29.71±0.47 °C for the 1st, 2nd, 3rd, 4th, and 5th larval sample time, respectively. As

the soil dried, the larvae crawled out of the hanging bags and fell into plastic pans (35.5

cm diam.) filled with ~ 5 cm of water. Larvae were collected at least twice a day and

were stored in 95% ethanol until they could be processed. During processing, each larva

recovered was closely examined for the presence of urogomphi, small appendages on

the posterior margin of the anal plate, which are only present on southern corn

rootworm (*Diabrotica undecimpuncta howardi* Barber) larvae (Krysan 1986). Any

southern corn rootworm larvae found were counted and discarded. The western corn

rootworm larvae from each sample were counted and head capsule width

measurements taken. In 2011, larval samples were not taken.

Plant Damage

Roots in subplots designated for damage evaluations were dug, washed, and

rated for damage using the node injury scale (Oleson et al. 2005). Two sets of subplots

were evaluated each year. In 2010, the first set was taken on June 30 (~700 degree

days) when damage to isoline roots had likely peaked based upon the number and size

of larvae recovered from the final larval sampling date 5 d earlier. The first western corn

rootworm beetles emerged on July 2, 2010. A second set of damage evaluations for all treatments were taken on July 15, 2010 (~950 degree days). The delay accounted for a potentially slower development of western corn rootworm larvae expected on the transgenic roots (Gray et al. 2007). In 2011, the first damage evaluation sample was taken July 11 (~530 degree days) and the second sample was taken later at July 25 (~670 degree days).

Adult Recovery

To ensure collection of emerging adults, emergence traps were placed over the corn on June 22, 2010 and June 23, 2011, which was well before the first predicted western corn rootworm adult emergence of ~700 degree days as calculated by degree day models (Wilde 1971, Levine et al. 1992). In 2010, emergence traps were placed over either the north or center plant of the three plant subplots. Because of plant spacing issues, only the north or center plant of each subplot was used and the number of adult emergence subplots was doubled to account for this. Emergence trap design was adapted from Hein et al. (1985) with modifications from Pierce and Gray (2007). Dimensions were 76.2 cm × 45.7 cm. Emergence traps consisted of a wooden frame covered in wire mesh with two holes cut into the center wooden support where the plant is pulled though one hole and tied off using a gauze sock and cable tie. A funnel was placed into the second hole and a jar fitted opening side down over the funnel. A metal trim protruded below the wooden frame ~5 cm into the soil. The long portion of each trap always protruded halfway into each inter-row (Hein et al. 1985), except in

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2011 for the blended plots where the center plant was sampled in which the long end of each trap was parallel with the row (Fig. 2). In the 2010 plots, adult emergence subplots had only one plant that was infested per subplot for all treatments (the north end or center plants), due to insufficient numbers of eggs. This also allowed sampling of the center plants in such a way that any excess portion of the emergence cage was on the south side in 2010, which was not infested that year (Fig. 2). In 2011, a sufficient amount of eggs were attained which allowed for both the center or the north and south plants in the adult subplots to be infested for treatments five through eight. In all situations, emergence traps were situated such that they protruded into the zone of the other subplot plants as little as possible (Fig. 2). Adult emergence traps were kept over the plants until two weeks after the last adult was collected. Both southern corn rootworm, *D. undecimpunctata howardi* Barber, and western corn rootworm were collected two to three times a week for the duration of the adult sampling period. Southern corn rootworm beetles were counted and discarded. Total number, head capsule width, sex, and dry weight of western corn rootworm beetles recovered from each plant were recorded.

Statistical Analysis

An ANOVA was conducted for the data analysis using PROC MIXED of the SAS statistical package (SAS Institute 2008). The random effects in the mixed model were treatment, replication and sample time and the fixed effect was plant. For larval recovery, larval dry weight, and plant damage the linear statistical model contained the

main plot effect of treatment, the subplot effect of sample, the subsub plot of plant (center or end plant), and all possible interactions. Data from the two end plants of each plot were averaged prior to analysis. Replication × treatment was the denominator of F to test treatment. Replication within treatment and sample date was the denominator for sample time and treatment×sample time. Plant and all other effects used the residual error for the denominator of F. Although the tables show the untransformed data, data were transformed by square root (x+0.5) to meet the assumptions of the analysis. Beyond the standard ANOVA, we conducted preplanned comparisons of treatment means within sample times and between sample times within treatment. This was done with the t-test output from PROC MIXED. For beetle emergence and beetle average dry weight, the linear statistical model contained the main plot effect of treatment and the subplot of plant (center or end plant), and the interaction of treatment×plant. This was done with the t-test output from PROC MIXED. Beetle emergence data were further analyzed by estimating the ordinal date (sometimes called Julian date) for 50% beetle emergence among plants within each treatment and the 95% confidence interval of this point. Data were averaged across replications and beetle sex. Treatments 1 and 5, with all SmartStax plants, were excluded from the adult weight and head capsule width data analysis because too few beetles emerged from these treatments. Maximum-likelihood estimates of regression were calculated using the PROC PROBIT of the SAS statistical package (SAS Institute 2008) was used to calculate 50% emergence from observed cumulative emergence both years in ordinal dates.

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Finally, a generalized linear mixed model was used to analyze sex ratio of the beetles produced from each treatment and plant using the PROC GLIMMIX logit link function and a binomial distribution. Since the total number of beetles emerged from SmartStax corn was small, a factor of 0.0001 was added to the total beetles from each single plant emergence cage to enhance convergence of the analysis.

Results

Larval recovery

The number of larvae recovered from the SmartStax plant from Treatment 8, which was surrounded by two, infested isoline plants (Fig. 1), was significantly greater on the later sample dates than the first sample date (Tables 1, 2), documenting significant larval movement from isoline plants to SmartStax plants. The only other SmartStax plants with similar data were the end plants from Treatment 3 which were also adjacent to an infested isoline plant (Fig. 1, Table 2). Larval recovery data from other plants indicated western corn rootworm larvae also moved from infested SmartStax plants to neighboring isoline plants. The number of larvae recovered from isoline plants adjacent to infested SmartStax plants in Treatments 4 and 7 (Fig. 1) increased significantly from the first to third sample date while the number of larvae recovered from the infested SmartStax plant in the same treatment did not increase significantly (Tables 1, 2). In each of these two treatments, western corn rootworm larvae were required to move through a SmartStax root system before encountering the

isoline plant (Fig. 1). Overall, the number of larvae recovered on the third sample date was significantly greater than the number of larvae recovered from all other sample dates when data for all treatments and plants were combined (Table 2). The date with the fewest number of larvae recovered was the final sample date (i.e. the fifth sample), when many of the larvae had pupated (the first western corn rootworm beetles were collected from this experiment just three days later).

Larval head capsule widths differed between treatments and between sample dates (Table 1 and 3). The infested Bt plant from Treatment 1 that was surrounded by uninfested Bt plants had the smallest head capsule widths overall when all sample dates were combined (Table 3). In Treatment 3, the infested, center isoline plant had smaller head capsule widths than the uninfested surrounding Bt plants (Table 3), yet there was no significant difference overall between the infested, center Bt plant and the surrounding uninfested isoline plants in Treatment 4 (Table 3). There was a significant difference in the overall head capsule widths of larvae recovered from the uninfested center isoline plant and the infested Bt plant of Treatment 7, yet there was no significant difference between the uninfested center Bt plant and the surrounding infested isoline plants in Treatment 8 (Table 3).

Plant Damage

The overall level of damage in 2010 to isoline plants was greater than damage in 2011, although most trends were similar across both years (Fig. 3). In 2010, plant damage ratings of the SmartStax plants were significantly lower than damage ratings of

all infested isoline plants on the second sample date except in Treatment 8 when a SmartStax plant was surrounded by two infested, highly-damaged isoline plants (Fig. 3A, Table 1). The Treatment 8 SmartStax plants were not significantly more damaged than any other SmartStax plant in any treatment on the first sample date, but on the second sample date, these plants were significantly more damaged than all other SmartStax plants (Fig. 3A). Apparently, this damage occurred later in the season than most of the damage to isoline plants. Treatments 5, 6, 7, and 8 were infested on the sides of the end plants away from the center plant of the three-plant subplot (Fig. 1), so any western corn rootworm larval damage found on the center plant was likely the result of larval movement from the infested end plants. Since the overall number of southern corn rootworm beetles recovered from adult emergence subplots was significant (45% of all beetles in 2010 and 56% in 2011) and there was no significant difference between treatments in terms of the number of southern corn rootworm beetles recovered (see below), we must assume that some of the damage seen in Fig. 3 is due to feeding from southern corn rootworm larvae and that this damage was evenly distributed among treatments. Trends in 2011 were similar to the 2010 data (though with less overall damage) with the exception that the uninfested Bt plant in Treatment 8 had significantly less damage than the surrounding infested isoline plants on both sample dates (Fig. 3B, Table 1).

Adult Recovery

Western corn rootworm. Overall, the number of western corn rootworm beetles recovered from the SmartStax plants was low compared with the number of beetles recovered from the isoline plants in both years of the study (Fig. 4). When a seed blend including isoline plants was included among the three-plant plot, nominally more western corn rootworm beetles always emerged than in plots with just SmartStax plants (Fig. 4). In 2010, both treatments where a SmartStax plant was surrounded by two isoline plants (Treatments 4 and 8) produced significantly more beetles from the SmartStax plant than SmartStax plots without any isoline plants (Fig. 4A). In fact, the SmartStax plant in Treatment 8 produced significantly more western corn rootworm beetles than emerged from any other plant in any treatment in 2010 (Fig. 4A). Egg placement forced any western corn rootworm beetles found on this plant to move through the roots of an isoline plant prior to reaching the SmartStax plant (Fig. 1). In 2010 and 2011, beetle emergence from Treatment 7 (the isoline plant surrounded by two infested Bt plants), where larvae were forced to move through a SmartStax plant prior to any potential movement to the center isoline plant (Fig. 1) was not significantly different than beetle emergence from Treatment 1 or 5, where all plants were SmartStax (Fig. 4). Western corn rootworm beetle emergence from isoline plants depended upon which plants were infested and which plants were adjacent (Fig. 4).

In 2010 and 2011, the ratio of males to females recovered from the adult emergence subplots did not differ significantly between treatments, plants, or in the

interaction of treatment×plant (Table 1). Average head capsule width of beetles did not differ significantly between treatments, plant within treatment, or their interaction in 2010 and 2011 (Table 1). Adult dry weight was significantly impacted by the interaction of treatment by plant in 2010 and by treatment in 2011. Overall, adult dry weight was variable and patterns were not consistent between years (Fig. 5).

Time in ordinal days to 50% beetle emergence in 2010 for both plant types in Treatment 7 was significantly delayed in relation to most other treatments including both plant types for Treatments 2, 4, 6, and 8 as indicted by non-overlapping 95% confidence intervals (Table 4). In 2011, the time to 50% emergence for uninfested end Bt plants for Treatment 3 occurred at an ordinal date of 201.15 (95% CI 198.74 to 203.46) which was a significant delay from all other treatments as indicated by the non-overlapping 95% confidence intervals (Table 4). Beetle emergence from straight SmartStax subplots (Treatments 1 and 5) in both years and the infested Bt plant of Treatment 7 in 2011 was too low for accurate calculation of 50% emergence.

Southern corn rootworm. The overall number of southern corn rootworm beetles recovered from the emergences traps was large, accounting for 45% of the total beetle emergence in 2010 and 56% of total emergence in 2011. In both years, there was no significant difference found in the number of southern corn rootworm beetles recovered between treatments, plant within treatment, or their interaction (Table 4), suggesting that SmartStax was not effective in managing the southern corn rootworm under the conditions of this experiment.

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Discussion

As in all previous research focused on post-establishment larval movement by western corn rootworm (Strnad and Bergman 1987b, Hibbard et al. 2003, 2004, 2005), movement from plant to plant also occurs in SmartStax seed blend scenarios (Figs. 3 - 4). In susceptible (non-Bt) corn, western corn rootworm larvae will initially establish on roots that are available near where they hatch and then move to younger nodes of roots as they emerge from the side of the stalk when larvae are older (Strnad and Bergman 1987b). These newly emerging nodal roots are not only preferred by older western corn rootworm larvae, but may also be required for completion of development to the adult stage (Hibbard et al. 2008a, 2009, Frank et al. 2011). In the current study, significantly more western corn rootworm beetles emerged from an uninfested SmartStax plant which was adjacent to two isoline plants than any other plant from any treatment in 2010 (Fig. 4A). Plants from this same treatment were the only SmartStax plants with damage ratings that did not differ significantly from most of the isoline plants on the second damage sample date in 2010 (Fig. 3A), although these plants had much less damage than isoline plants on the first sample date. Overall, western corn rootworm larval movement from isoline plants to SmartStax plants was clearly documented (Table 1, 2, Figs. 3-4), though the plant configuration where damage to and emergence from SmartStax was the highest (in 2010 in Treatment 8) would occur only 0.24% of the time in a 5% seed blend and 0.9% of the time in a 10% seed blend. More larval movement between SmartStax and isoline corn and vice versa appeared to take place than in a

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similar study between Cry3Bb1 plants and isoline plants conducted in 2001 and 2002

(Hibbard et al. 2005). In that study, larval movement from isoline to Cry3Bb1 was not

detected in larval sampling and apparently occurred later than the current study

because it was primarily picked up during the second sample date for plant damage. In

addition to movement from isoline to SmartStax plants, significantly more larvae were

recovered from uninfested isoline plants adjacent to infested SmartStax plants on the

third sample date than on the first sample date for both Treatments 4 and 7,

documenting that western corn rootworm larvae also moved from SmartStax plants to

isoline plants (Table 2). Overall, movement by western corn rootworm larvae clearly

took place in both directions, but adult emergence from and damage to SmartStax

plants was not as great in 2011.

Overall, damage did not exceed an average rating of 1.2 in 2010 or 0.8 in 2011

on the node injury scale (Oleson et al. 2005) for any plant (Fig. 3), so damage was not

extreme even to isoline plants in this trial. In fact, with damage ratings less than 0.8 in

2011, density-dependent mortality was likely low (Hibbard et al. 2010a) and movement

to SmartStax was likely not forced by larvae searching for food (Hibbard et al. 2004).

Plants expressing Cry3Bb1 (and perhaps Cry34/35Ab1) are also less preferred by

western corn rootworm larvae than isoline corn (Clark et al. 2006), and movement to

these plants would be expected to be less than to isoline plants (Hibbard et al. 2005).

The main criteria for whether movement by western corn rootworm larvae

between isoline and SmartStax plants will influence the development of resistance is

whether or not selection for resistance is taking place. If, for instance, all larvae that moved from isoline to SmartStax plants were third instars, and all survived this movement because third instar larvae can tolerate the levels of Cry proteins in SmartStax plants, then selection for SmartStax resistance would be minimal and the effect on resistance management would be primarily positive (additional susceptible beetles would be emerging from within the SmartStax field). For plants expressing Cry34/34Ab1, survivorship to the adult stage of third instars (reared previously on isoline corn) was 65% as compared to 0.5% survivorship of neonate larvae to the adult stage (Binning et al. 2010), supporting the suggestion of a reduced effect of late larval movement from isoline to transgenic corn on selection. As indicated in Tables 1 and 2, the likely time frame that many of the larvae moved from infested isoline plants to SmartStax plants in Treatment 8 was between the 2nd and 3rd larval sample dates. The range of head capsule widths of second instar larvae on susceptible corn was between 0.30 and 0.38 mm (Hammack et al. 2003). Larvae recovered from SmartStax plants on the third sample date averaged 0.38 mm for Treatment 8 (Table 2), so it was likely a mixture of second and third instar larvae that moved, but with more second than third instars.

In Meihls et al. (2008), when western corn rootworm larvae were reared on isoline corn for 1 wk and then reared on Cry3Bb1 corn (Late exposure colony) for the remainder of larval development, this colony did develop resistance, but it developed more slowly than larvae that were reared completely on Cry3Bb1 expressing corn

(Constant exposure colony). Larvae that were exposed to Cry3Bb1 corn, but could crawl off and finish their development on isoline corn (the Neonate exposure colony) did not develop resistance when assayed in a no-choice experiment with only Cry3Bb1 corn (Meihls et al. 2008). Binning et al. (2010) showed that neonate survival on Cry34/35Ab1 corn was approximately 33% of isoline survival after 17 d, and the same 33% recovered and developed to adulthood when they were transferred to isoline corn. After 17 d on Cry34/35Ab1 or isoline the percentage of larvae that were 1st, 2nd, or 3rd instars was 61, 36, and 3% on Cry34/35Ab1 and 1, 15, and 84% on isoline corn. This difference has been suggested as a monitoring tool to detect resistance (Nowatzki et al. 2008). It is unclear how the neonate exposure selection scheme of Meihls et al. (2008) or Binning et al. (2010) relates to larvae that initially developed on Bt corn and then moved to isoline corn in Treatment 4 and 7 of the current experiment. Recently moved larvae in the current experiment were recovered on the lower end of the second instar head capsule width natural variability, averaging 0.34 mm for Treatment 4 and 0.32 mm for Treatment 7 (Table 2). More larvae were also 2nd instars than in Binning et al. (2010). It is uncertain to what degree, if any, resistance would develop in those larvae exposed to the Bt toxins for longer periods.

One of the charges for the December, 2010 EPA Scientific Advisory Panel, which considered issues associated with a potential SmartStax seed blend refuge, concerned the percentage of males emerging in a seed blend situation (EPA 2011a). Apparently, reduced male emergence had been found in some seed blend situations. Based on data

they were provided, the EPA Scientific Advisory Panel also concluded that males produced from SmartStax 5% refuge in a bag may be less fit than those produced from non-seed blend fields (EPA 2011a). In the current study, there was no significant difference in percent male emergence between treatments, plants, or in the interaction of treatment and plant (Table 3), so reduced male emergence was not an issue under our conditions. Average adult head capsule width of beetles did not differ significantly between treatments, plant within treatment, or their interaction for either year (Table 3) suggesting equal fitness of beetles emerging SmartStax and isoline.

The urogomphi trait of the southern corn rootworm larvae is not always present (Hibbard et al. 2005). The proportion of southern corn rootworm larvae identified versus western corn rootworm larvae, as indicated by larvae with urogomphi, was smaller than the proportion of southern corn rootworm adults recovered versus western corn rootworm adults recovered. This indicates that some of the larvae in Tables 1 and 2 were likely southern corn rootworms. The difference may not have affected the results overall because the amount of southern adults recovered did not differ between treatments. Given that the number of southern corn rootworm beetles that emerged during both years of the study was quite substantial, it is possible that larval-larval competition influenced the results in some way. Since southern corn rootworm beetle emergence did not differ between treatments and they emerged earlier than western corn rootworm beetles, on average, for this experiment, it is also possible that southern corn rootworm larvae opened up access to portions of the root

that express lower levels of Bt (it is known that protein expression, including Bt is expressed to a greater extent on the outside of roots).

In summary, western corn rootworm larvae will move from isoline to transgenic and transgenic to isoline in SmartStax seed blend scenarios. In rare situations where a SmartStax plant is surrounded by two isoline plants, late western corn rootworm larval movement to SmartStax plants may produce significantly increased damage ratings and beetle emergence compared to SmartStax plants surrounded by SmartStax plants. In general, though, damage to and beetle emergence from SmartStax plants in the most common seed blend scenarios were not significantly different than damage and beetle emergence in pure-stand SmartStax plots. The 2010 EPA Scientific Panel concluded that a 5% SmartStax seed blend would have comparable durability to SmartStax planted with a 5% structured refuge for western corn rootworm resistance management (EPA 2011a). We can find nothing in the current study related to larval movement that would refute that conclusion. Selection of insect colonies using seed blends may be needed to assess their long-term success.

Acknowledgements

Table 1. Factors influencing western corn rootworm larvae (Proc mixed tables for the no. of larvae recovered, larval average head capsule width (HCW), percent of males, no. of adults recovered, adult dry weight(g), adult average HCW and root damage rating) of the rootworms recovered from the corn field in 2010 and 2011

Analysis	Effect	2010			2011		
		df	f	P			
No. larvae	Samptime	4,128	33.71	<0.0001	-	-	-
	Trt	7,28	39.91	<0.0001	-	-	-
	Samptime×trt	28,128	2.90	<0.0001	-	-	-
	Plant	1,160	0.21	0.6455	-	-	-
	Samptime×plant	4,160	1.27	0.2855	-	-	-
	Trt × plant	7,160	32.11	<0.0001	-	-	-
	Samptime×trt×plant	28,160	4.77	<0.0001	-	-	-
Larvae HCW	Samptime	4,122	121.12	<0.0001	-	-	-
	Trt	7,28	3.93	0.0042	-	-	-
	Samptime×trt	28,122	1.33	0.1477	-	-	-
	Plant	1,112	4.31	0.0402	-	-	-
	Samptime×plant	4,112	0.43	0.7842	-	-	-
	Trt×plant	7,112	3.26	0.0034	-	-	-
	Samptime×trt×plant	28,112	2.46	0.0005	-	-	-
WCR beetles	Trt	7,28	17.73	<0.0001	7,28	6.03	0.0002
	Plant	1,32	0.04	0.8466	1,32	1.88	0.1796
	Trt × plant	7,32	3.42	0.0077	7,32	0.75	0.6322
Percent males	Trt	5,20	1.29	0.3081	5,20	0.15	0.9768
	Plant	1,24	0.42	0.5207	1,24	0.00	0.9647
	Trt × plant	5,24	1.18	0.3473	5,24	0.72	0.6174
Adult weight	Trt	5,20	1.30	0.3040	5,16	3.58	0.0231
	Plant	1,19	0.09	0.7648	1,11	2.15	0.1704
	Trt×plant	5,19	3.24	0.0279	5,11	1.35	0.3156
Adult HCW	Trt	5,20	0.30	0.9075	5,16	0.40	0.8437
	Plant	1,19	0.59	0.4500	1,11	2.28	0.1591
	Trt×plant	5,19	1.59	0.2117	5,11	2.01	0.1560
Damage rating	Samptime	1,32	8.87	0.0055	1,29	0.13	0.7160
	Trt	7,28	32.18	<0.0001	7,28	17.21	<0.0001
	Samptime×trt	7,32	1.60	0.1699	7,29	0.60	0.7470
	Plant	1,64	0.02	0.8972	1,60	1.07	0.3057
	Samptime×plant	1,64	1.36	0.2473	1,60	0.22	0.6383
	Trt×plant	7,64	14.03	<0.0001	7,60	7.72	<0.0001
	Samptime×trt×plant	7,64	1.31	0.2611	7,60	0.30	0.9510
SCR beetles	Trt	7,28	1.96	0.0978	7,28	0.66	0.7481
	Plant	1,32	0.82	0.3706	1,32	1.05	0.1739
	Trt × plant	7,32	0.52	0.8119	7,32	1.40	0.2051

Table 2. Western corn rootworm larvae means±SE recovered from each treatment over five sample dates from the corn field in 2010

				Corn developmental stage at recovery					
Plant	Trtmt	Seed	Infest	V6	V8	V11	V12	VT	mean
Center	1	Bt	infest	4.0 ± 1.6aBCD	3.2 ± 1.2aCD	1.8 ± 1.3aEF	1.2 ± 0.6aCD	1.3 ± 0.6aBC	2.3±0.5E
End	1	Bt	not infest	1.3 ± 0.8aCD	2.8 ± 0.8aCD	1.9 ± 0.6aEF	1.2 ± 0.4aCD	0.7 ± 0.3aBC	1.6±0.3E
Center	2	Iso	Infest	24.0 ± 11.0cdAB	47.0 ± 7.6bA	75.0 ± 5.6aA	10.0 ± 4.1eAB	11.0 ± 1.4deAB	33.6±5.7A
End	2	Iso	not infest	5.8 ± 2.7bBCD	8.2 ± 2.3bBCD	26.0 ± 4.6aC	8.9 ± 2.9bB	8.4 ± 1.6bAB	11.5±1.7C
Center	3	Iso	Infest	13.0 ± 4.4bB	51.0 ± 23.0aA	39.0 ± 10.0aBC	11.0 ± 3.1bAB	8.4 ± 3.9bAB	24.5±5.9B
End	3	Bt	not infest	0.8 ± 0.3bCD	3.5 ± 1.0abCD	7.5 ± 3.5aDEF	3.6 ± 0.8abBCD	4.1 ± 0.8abBC	3.9±0.8DE
Center	4	Bt	Infest	4.4 ± 1.5aBCD	13.0 ± 5.0aBC	10.0 ± 3.0aDE	3.2 ± 0.6aBCD	3.4 ± 0.9aBC	6.8±1.4D
End	4	Iso	not infest	0.6 ± 0.3cCD	3.1 ± 1.1bcCD	18.0 ± 3.8aCD	6.5 ± 1.2bBC	5.1 ± 1.0bcBC	6.7±1.2D
Center	5	Bt	not infest	1.2 ± 1.0aCD	3.2 ± 1.8aCD	2.0 ± 1.5aEF	0.8 ± 0.5aCD	0.8 ± 0.4aBC	1.6±0.5E
End	5	Bt	Infest	4.9 ± 2.0abBCD	12.0 ± 4.0aBC	2.7 ± 0.62bEF	1.0 ± 0.3bCD	1.7 ± 0.6bBC	4.6±1.1DE
Center	6	Iso	not infest	2.8 ± 1.7cCD	15.0 ± 4.3bB	48.0 ± 13.0aB	17.0 ± 4.2bAB	16.0 ± 3.4bA	19.7±4.1B
End	6	Iso	Infest	32.0 ± 11.0bA	54.0 ± 13.0aA	65.0 ± 9.4aAB	14.0 ± 3.1cAB	9.4 ± 1.6cAB	35.0±4.9A
Center	7	Iso	not infest	1.2 ± 0.5bCD	5.6 ± 3.7abCD	7.8 ± 2.9aDEF	5.0 ± 1.2abBCD	3.2 ± 1.1abBC	4.6±1.0DE
End	7	Bt	Infest	9.3 ± 4.3aBC	7.7 ± 2.4abBCD	4.9 ± 1.3abDEF	3.4 ± 1.1abBCD	1.4 ± 0.2bBC	5.4±1.1D
Center	8	Bt	not infest	1.6 ± 1.2bCD	5.2 ± 1.7abCD	13.0 ± 3.2aD	8.4 ± 4.4aB	13.0 ± 4.9aAB	8.2±1.7CD
End	8	Iso	Infest	32.0 ± 16.0bA	56.0 ± 13.0aA	54.0 ± 12.0aB	20.0 ± 5.8bcA	12.0 ± 2.4cAB	34.9±5.4A
mean				9.5±2.0c	18.2±2.6b	23.4±2.6a	7.3±0.9c	6.1±0.6c	

The lower case letters indicate significance within rows and uppercase letters significance within columns (*P*≤0.05) using Fisher's LSD test.

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Table 3. Western corn rootworm larval head capsule width (mm) means±SE of larvae recovered from each treatment over five sample dates from the corn field in 2010

Plant	Trtmt	Seed	Infest	Corn developmental stage at recovery					
				V6	V8	V11	V12	VT	mean
center	1	Bt	Infest	0.24±0.04abABC	0.23±0.05bAB	0.25±0.03abBC	0.27±0.07abC	0.31±0.08aBC	0.25±0.02C
end	1	Bt	not infest	0.19±0.01cBC	0.23±0.02cAB	0.29±0.04bBC	0.33±0.04bBC	0.48±0.01aA	0.30±0.02B
center	2	Iso	Infest	0.22±0.02bBC	0.22±0.02bAB	0.36±0.02aAB	0.41±0.01aAB	0.39±0.01aB	0.32±0.02AB
end	2	Iso	not infest	0.25±0.01bAB	0.27±0.02bAB	0.37±0.01aA	0.39±0.02aAB	0.41±0.01aAB	0.34±0.01A
center	3	Iso	Infest	0.19±0.02cBC	0.24±0.01bcAB	0.30±0.02bBC	0.34±0.02abBC	0.37±0.04aBC	0.30±0.02B
end	3	Bt	not infest	0.30±0.03bA	0.22±0.02cABC	0.33±0.02bAB	0.42±0.02aA	0.45±0.01aA	0.36±0.02A
center	4	Bt	Infest	0.17±0.01bBC	0.24±0.02bAB	0.35±0.02aAB	0.38±0.02aAB	0.41±0.02aAB	0.32±0.02AB
end	4	Iso	not infest	0.35±0.00abA	0.23±0.02cAB	0.34±0.02bAB	0.37±0.03abAB	0.41±0.02aAB	0.34±0.01A
center	5	Bt	not infest	0.26±0.04cAB	0.15±0.03dBC	0.28±0.01bcBC	0.38±0.07bAB	0.49±0.01aA	0.29±0.04B
end	5	Bt	Infest	0.22±0.01cBC	0.21±0.03cBC	0.33±0.04bAB	0.41±0.04aAB	0.39±0.05abBC	0.30±0.02B
center	6	Iso	not infest	0.22±0.01bBC	0.25±0.03bAB	0.38±0.02aA	0.40±0.02aAB	0.42±0.01aAB	0.36±0.02A
end	6	Iso	Infest	0.22±0.02cBC	0.28±0.03bA	0.35±0.01aAB	0.39±0.01aAB	0.42±0.01aAB	0.33±0.01A
center	7	Iso	not infest	0.24±0.03cdABC	0.25±0.03cdAB	0.32±0.02bcABC	0.35±0.03bB	0.44±0.03aA	0.33±0.02A
end	7	Bt	Infest	0.19±0.00cBC	0.18±0.02cBC	0.28±0.03bBC	0.40±0.03aAB	0.39±0.03aB	0.29±0.02BC
center	8	Bt	no infest	0.22±0.00bBC	0.23±0.02bAB	0.38±0.02aA	0.38±0.02aAB	0.40±0.00aAB	0.34±0.02A
end	8	Iso	Infest	0.21±0.01bBC	0.25±0.01bAB	0.36±0.01aAB	0.36±0.01aAB	0.41±0.01aAB	0.32±0.01AB
mean			Mean	0.22±0.01d	0.23±0.01d	0.33±0.01c	0.38±0.01b	0.41±0.01a	

The lower case letters indicate significance within rows and uppercase letters significance within columns ($P \leq 0.05$) using Fisher's LSD test.

Table 4. Ordinal dates for 50% emergence of adult western corn rootworm from the corn field in 2010(A) and 2011 (B).

A.

Plant	Treatment	Seed	Infest	50% Emergence - Ordinal Date	95% CI	
Center	2	Isoline	Infested	188.07	187.26	188.83
North	2	Isoline	Not Infested	189.05	188.26	189.78
Center	3	Isoline	Infested	191.43	189.39	193.22
North	3	Bt	Not Infested	191.38	190.30	192.40
Center	4	Bt	Infested	188.09	186.67	189.36
North	4	Isoline	Not Infested	190.58	189.70	191.39
Center	6	Isoline	Not Infested	189.05	187.95	190.07
North	6	Isoline	Infested	188.85	188.00	189.64
Center	7	Isoline	Not Infested	194.23	191.95	196.58
North	7	Bt	Infested	194.39	192.72	196.08
Center	8	Bt	Not Infested	188.61	188.00	189.20
North	8	Isoline	Infested	189.88	188.99	190.72

B.

Plant	Treatment	Seed	Infest	50% Emergence - Ordinal Date	95% CI	
Center	2	Isoline	Infested	193.94	193.20	194.67
North	2	Isoline	Not Infested	193.53	192.93	194.12
Center	3	Isoline	Infested	197.27	195.72	198.90
North	3	Bt	Not Infested	201.15	198.74	203.46
Center	4	Bt	Infested	194.45	190.06	198.11
North	4	Isoline	Not Infested	194.45	192.86	195.84
Center	6	Isoline	Not Infested	196.09	194.22	197.71
North	6	Isoline	Infested	194.88	194.24	195.52
Center	7	Isoline	Not Infested	195.40	194.80	196.01
North	7	Bt	Infested	196.99	.	.
Center	8	Bt	Not Infested	195.33	194.29	196.48
North	8	Isoline	Infested	195.45	194.60	196.32
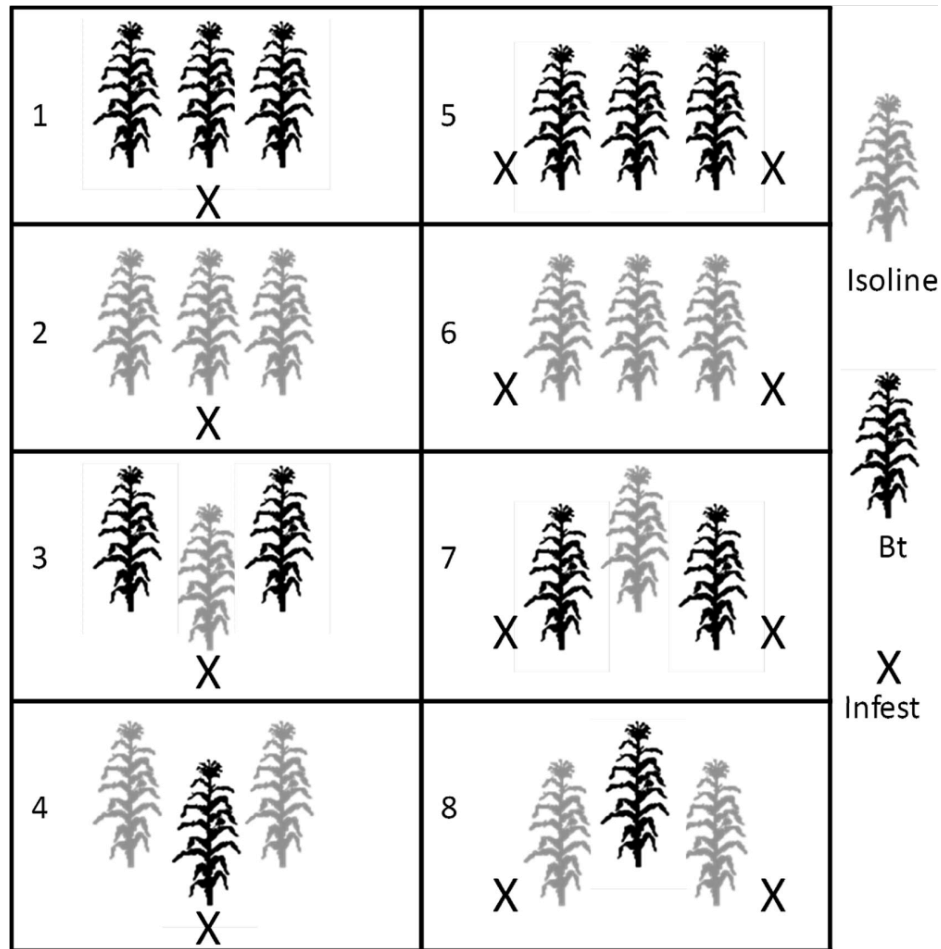
Figure 1. Planting and infestation combinations used in the eight treatments. In Treatments 1-4 the center plant was infested with western corn rootworm eggs and in Treatments 5-8 the end plants were infested with the exception of the adult recovery subplots in 2010 where only one end plant was infested. In Treatment 3 and 4 the middle seed was planted slightly off center so the larvae, once hatched, would have to travel through the roots to reach another plant.
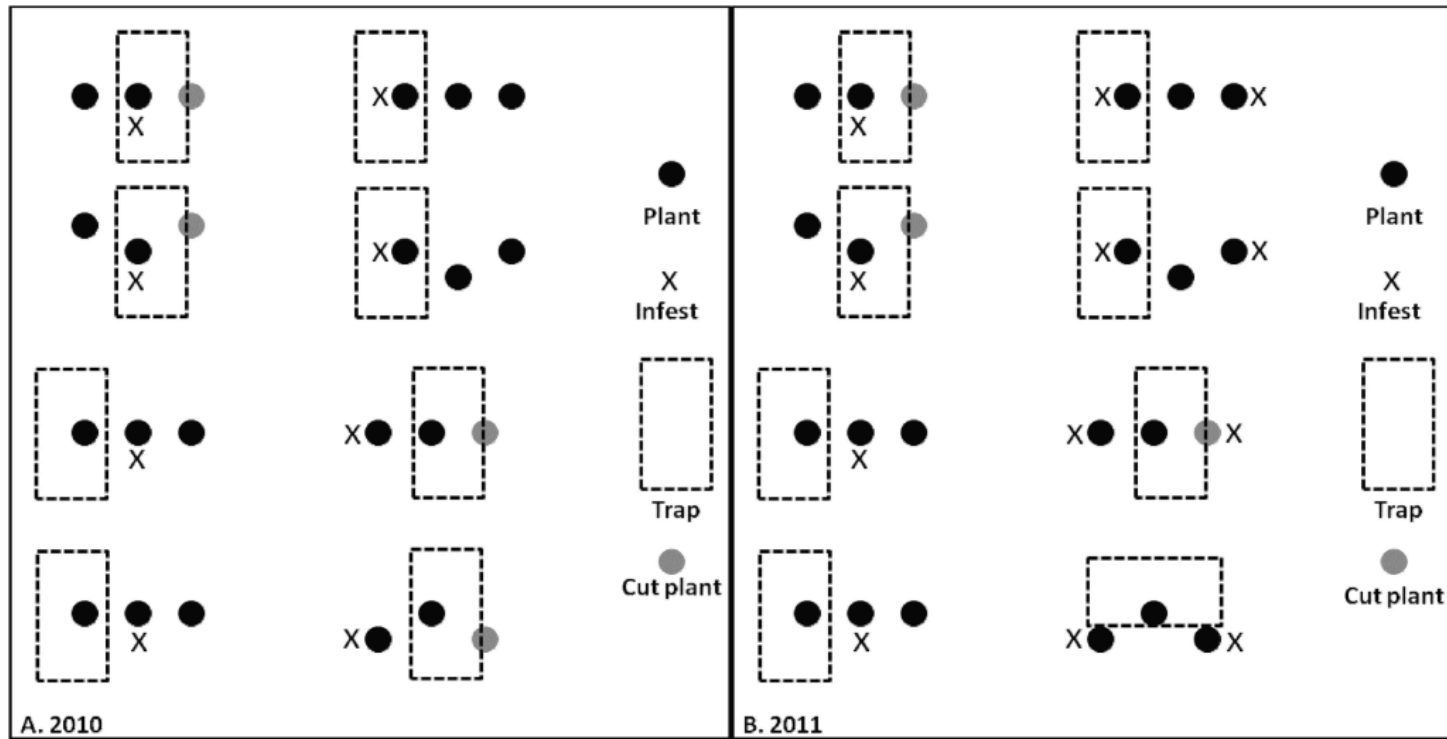
Figure 2. The plant configuration of the eight treatments in 2010 (A) and 2011 (B). In 2010, only one plant was infested, either the center or the north plant. In 2011, in treatments 1-4, the center plant was infested, while in Treatments 5-8 both ends plants where infested. Dimensions of the trap were 76.2 cm × 45.7 cm and the plant spacing is 19.05 cm. In some subplots where the emergence trap was placed over the center plant, the end plant above ground portion was destroyed to accommodate the size of the emergence trap.

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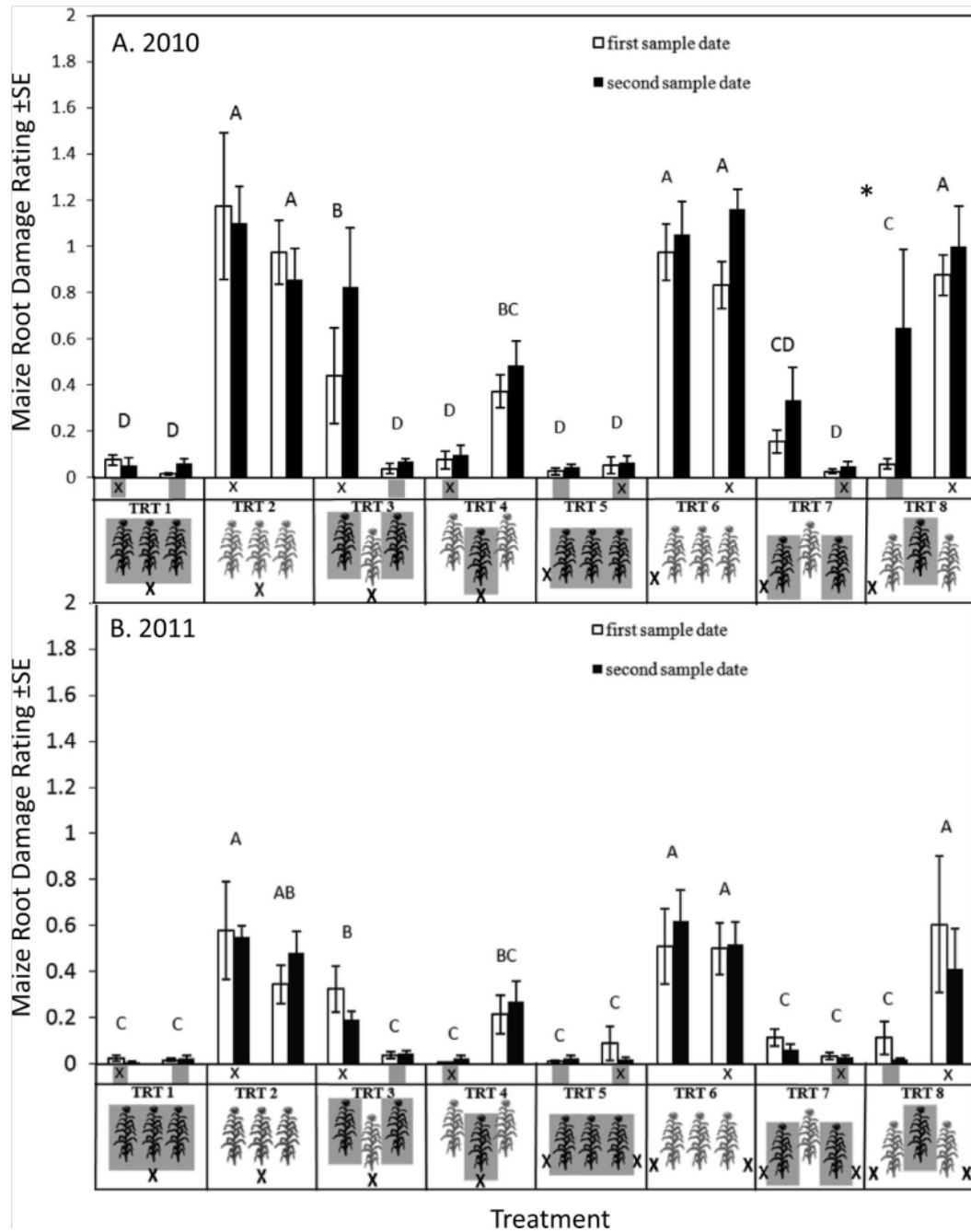
Figure 3. Mean±SE damage rating from two sampling periods in eight treatments of SmartStax and isoline corn plants from the corn field in 2010 (A) and 2011 (B). The gray boxes with black corn indicate SmartStax plants, the gray corn symbols indicate isoline plants and the X signifies the infested plants. The two end plants in each treatment were combined for each subplot. Different letters indicate significant differences between treatments (*P* ≤0.05). The only significant difference found between sample times.
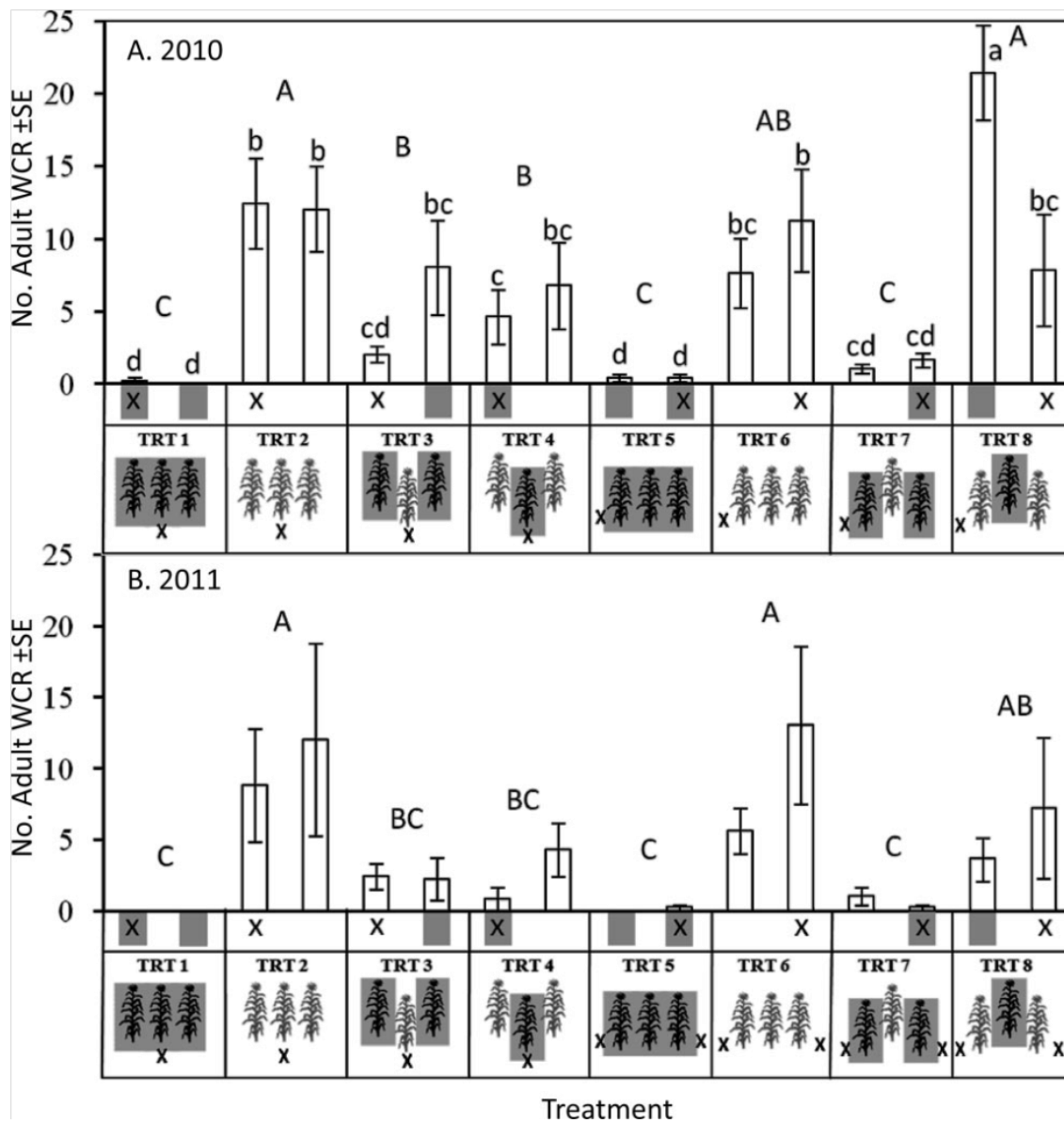
Figure 4. Mean±SE number of adult western corn rootworms recovered in the emergence traps in eight treatments from the corn field in 2010 (A) and 2011 (B). The gray boxes with black corn indicate SmartStax plants, the gray corn symbols indicate Isoline plants and the X signifies the infested plants. The two end plants in each treatment were combined. Different letters indicate significant differences between treatments (*P* ≤0.05). Uppercase is indicates differences between within treatments, lowercase indicates differences between plants (2010 only).
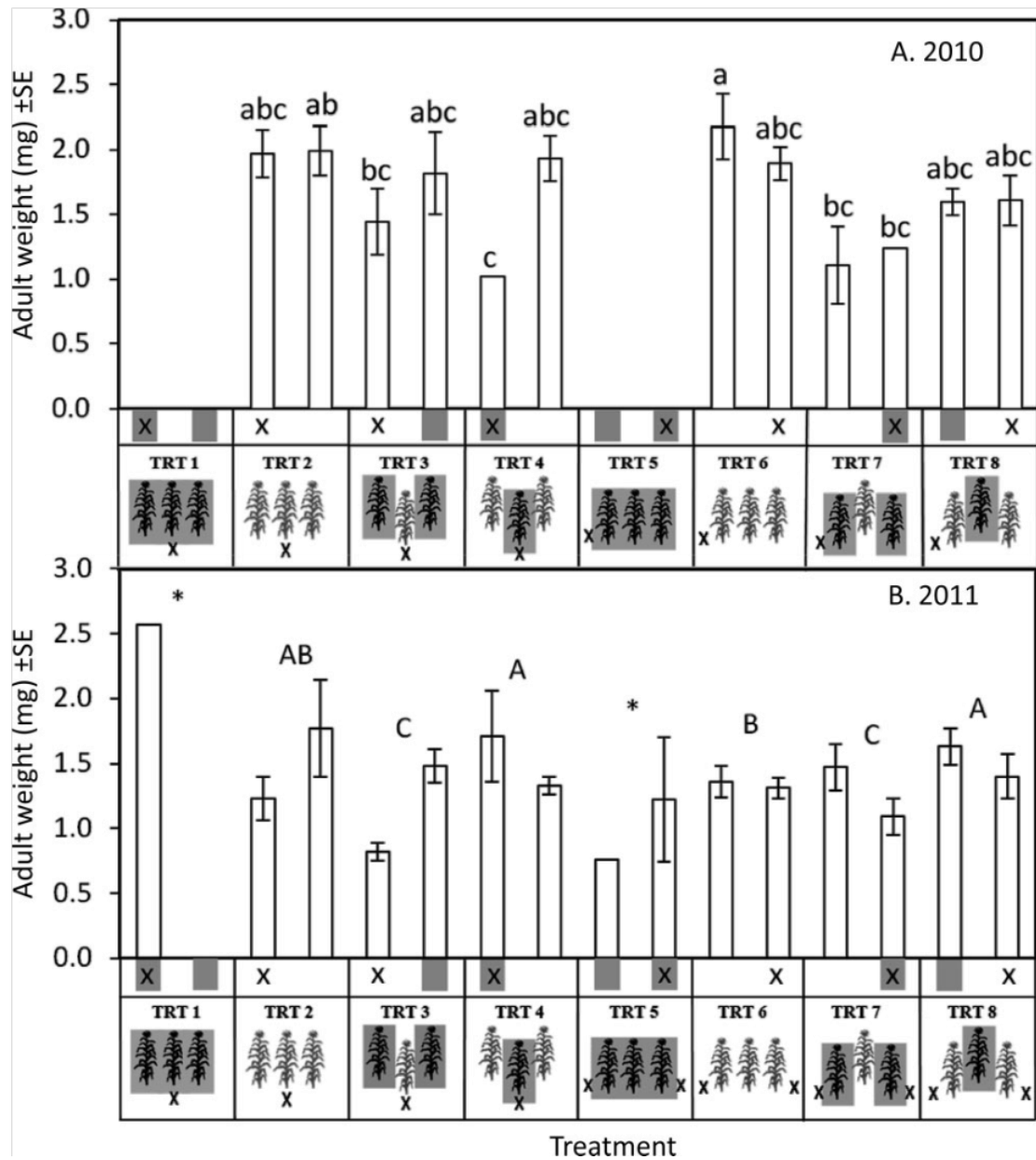
Figure 5. Mean±SE weight of adult western corn rootworm recovered in the emergence traps in eight treatments from the corn field in 2010 (A) and 2011 (B). The gray boxes with black corn indicate SmartStax plants, the gray corn symbols indicate Isoline plants and the X signifies the infested plants. The two end plants in each treatment were combined. Different letters indicate a significant difference (*P* ≤0.05). Lowercase letters indicate differences between plants (2010), uppercase letters indicates differences between treatments (2011). Treatment was not significant for 2010 and plant × treatment interaction was not significant for 2011. *In 2010 and 2011, Treatment 1 and 5 were dropped from the analysis due to too few beetles.

CHAPTER III: WESTERN CORN ROOTWORM LARVAL MOVEMENT IN DURACADE SEED BLEND SCENARIOS

Introduction

Corn (*Zea mays* L.) incorporating *Bacillus thuringiensis* (Bt) proteins have been widely used to control above ground pests since their introduction in 1996, and below ground pests such as the western corn rootworm (WCR), *Diabrotica virgifera virgifera* Leconte, since 2003. Out of all Bt planted in the U.S., Bt corn hybrids account for > 65% of the acreage planted in 2011 (Fernandez-Cornejo and Wechsler 2012). The Environmental Protection Agency (EPA) mandates that all Bt hybrids include an Insect Resistance Management (IRM) plan (EPA 1998). This non-Bt corn refuge, in theory, is used to maintain the pest's susceptibility to the Bt product and is considered essential if Bt technology is to persist (Matten et al. 2012). The purpose of this refuge is to produce susceptible insects emerging from isoline corn plants that will be available to mate with any insects that survive the Bt plants, thereby delaying the evolution of resistance, assuming resistance is recessive (EPA 1998). Bt technology has been successfully used to manage WCR until recently when higher than expected damage to Cry3Bb1-expressing corn has been discovered in places (Gassmann et al. 2011, Gassmann and Hodgson 2012, Porter et al. 2012).

Until recently, all IRM plans for Bt corn hybrids targeting the WCR in the past have required a 20% refuge in the Corn Belt. However, a reduction in refuge size to 5% was approved by the EPA for pyramided proteins in 2009. Pyramid hybrids incorporate

two or more Bt toxins that target the same pest, and the EPA has currently registered

three such pyramided products targeting WCR. Monsanto Company/DowAgro Sciences

have SmartStax® RIB Complete which not only has a reduced refuge of 5%, but also

incorporates a new refuge in a bag (RIB) strategy in which the refuge seeds are blended

in with the Bt seeds in the same bag (EPA 2011b). SmartStax incorporates two Bt

proteins, Cry34/35Ab1 (event DAS59122-7) and Cry3Bb1 (event MON88017) targeting

WCR. Optimum AcreMax (Pioneer) as well as Agrisure E-Z Refuge (Syngenta) are also

available products that have RIB and a reduced refuge requirement. Each of these

hybrids have Cry34/35Ab1 (event DAS59122-7) and mCry3a (event MIR604). Syngenta's

next-generation corn hybrid, Agrisure Duracade™, containing the eCry3.1Ab (event

5307) and mCry3a (event MIR604) rootworm-targeted toxins is expected to be

commercially available by 2014 pending final regulatory approval by the USDA. It has

already been approved by the EPA and FDA. This pyramided hybrid is expected to have a

blended 5% RIB as well. As refuge compliance has been an issue in some areas (Jaffe

2009, Gray 2011a, Gray 2011b), blended refuge strategies will eliminate this problem

when used (EPA SAP 2011). Another positive aspect of a RIB IRM plan is the ease of

mating between male susceptible insects and females that survive the Bt (Spencer et al.

2013). Males may be reproductively active for as little as 10 days and move only ~15

meters/day (Spencer et al. 2009, Pan et al. 2011). Reaching females emerging from the

Bt would probably be difficult in a larger field (Spencer et al. 2013).

As part of the IRM plan, resistance monitoring and having a complete

understanding of the biological parameters of a pest are essential in staying ahead of

resistance. Blended refuges will have obvious benefits for the farmer in not having to clean out the planter to plant refuge seed, but concern has been raised about rootworm larval movement and survival and what effect these parameters will have on resistance management in these RIB fields (EPA SAP 2011). WCR post-establishment larval movement has been documented previously, showing that larvae are capable of moving up to three plants down the row and up to 0.46 meters across the row (Hibbard et al. 2003). Hibbard et al. (2005) found that larvae can move from infested isoline plants to neighboring Bt plants in the field. Larval movement between isoline and Bt plants has also been shown to occur in the field in RIB scenarios, in which older larvae were shown to move from surrounding isoline plants to a center Bt plant later in the season and cause greater than expected damage to that Bt plant (Zukoff et al. 2012). The objective of this study was to assess larval movement, survival and root injury in RIB scenarios using the Duracade, eCry3.1Ab+mCry3A, hybrid seed.

Methods

The experiment was conducted at the Bradford Research and Extension Center ~9 km east of Columbia, MO. The field had been planted with soybeans (*Glycine max* L.) the previous year, therefore, it was assumed to not have a background WCR population because central Missouri does not yet have the rotation-resistant population (Gray et al. 2009). Overall, methods were similar to Zukoff et al. (2012). The experiment was planted on 26 April 2012. The experimental unit for this study was a subplot consisting of three consecutive corn plants each spaced approximately 15 cm apart. The

experimental design was a randomized complete block arranged in a split-split-plot

(larval recovery and plant damage) or a split-plot design (beetle emergence) as outlined

in Steel et al. (1997) in which the factors were arranged by a 8 × 5 × 2 for larval recovery

(treatment × sample date × plant), 8 × 2 × 2 for plant damage (treatment × sample date

× plant), and 8 × 2 for beetle emergence (treatment × plant). The eight treatments

consisted of four combinations of Bt and/or isoline corn along with two locations for

rootworm egg infestations (at either the center plant or at both end plants) with five

replications each. Each subplot had three consecutive plants (Fig. 6) that were either Bt

only (treatments 1 and 5), isoline only (treatments 2 and 6), an isoline center plant

surrounded by two Bt plants (treatments 3 and 7), or a Bt center plant surrounded by

two isoline plants (treatments 4 and 8). The isoline-only treatments acted as the block

refuge control, and the other treatments were the blended refuge scenarios. Each

three-plant subplot was either three plants in a straight row (treatments 1, 2, 5, and 6)

or three plants with the center plant (a kernel of the opposite type from the end plants)

slightly offset from the row (treatments 3, 4, 7, and 8 – see Fig. 6). Each replication

included each of the nine sample types that were randomized within each of the eight

treatments. Each replication of each treatment included subplots for plant damage (at

two different evaluation times), adult emergence (two subplots needed because plant

spacing did not allow sampling multiple plants within a subplot), and larval recovery at

five different recovery times. All larval sampling and planting methods were modified

from Hibbard et al. (2004) and Zukoff et al. (2012).

Between each 1.5 m plot, there were nine kernels of buffer corn (Pioneer 33M 16) planted in 1.5 m. Two buffer rows were also planted on each side of the field. Bulk seeds were planted by machine and all Bt and isoline seeds were hand planted. Each replication contained 72 plots and each row was 82.3 m long. Gene check strips (EnviroLogix, Portland, ME) were used to verify that all 540 Duracade plants expressed both the mCry3A and eCry3.1Ab toxins. All isolines in mixed treatments were also evaluated for both mCry3A and eCry3.1Ab. All gene checks confirmed the presence of the targeted genes where they should have been and absence where they were not supposed to be.

For treatments 1, 2, 3, and 4 the center plant of each subplot was infested and for treatments 5, 6, 7, and 8, the two end plants were infested (Fig. 6). The location of infestation was chosen so that larvae were forced to move through the infested plant before reaching any other plants in the subplot (with the possible exception of treatments 1 and 2 because they were planted all in a row – see Fig. 6). Approximately 1,700 western corn rootworm eggs were used for each infested plant. Viability averaged 80.0%, so there were ~1,300 viable eggs per plant infested. Natural western corn rootworm egg infestations of 12.2×10^7 eggs per ha have been documented, which is ~2,800 eggs per 30.5 cm of maize row (Pierce and Gray 2006). The amount of damage that 1,300 viable eggs typically cause is equivalent to a moderate to moderate/high infestation (Hibbard et al. 2004). Eggs were placed ~10 cm deep and ~2.5 cm from the plant base. Plants were infested at V2-3 (Ritchie et al. 1992) on 15 May 2012. Eggs were

obtained from the primary diapausing strain from the USDA-ARS laboratory in
Brookings, SD.

Larval Recovery

Larvae were sampled on five sample dates with the first sample taken on June 8,
330 degree days after infestation, when approximately 90% egg hatch had occurred and
subsequently every 4 to 5 days after. All three plants in each subplot were destructively
sampled. Using techniques similar to Hibbard et al. (2004), the top of the plant was cut
~30 cm from the ground, the root ball and surrounding soil were extracted with the aid
of a long handled drain spade, and each root ball was carefully placed in a mesh bag in
an attempt to keep the soil structure intact. The mesh bags containing roots, soil, and
larvae were hung in the greenhouse with the cooling system turned off for
approximately one week. Afternoon temperatures in the greenhouse averaged 38.3±1.5
°C from 13:00 to 16:00 h for all larval sample dates. As the soil dried, the larvae crawled
out of the hanging bags and fell into plastic pans (35.5 cm diam.) filled with ~ 5 cm of
water. Larvae were collected at least twice a day and were stored in 95% ethanol until
they could be processed. During processing, each larva recovered was closely examined
for the presence of urogomphi, small appendages on the posterior margin of the anal
plate, which are only present on southern corn rootworm (*Diabrotica undecimpuncta
howardi* Barber) larvae (Krysan 1986). The western corn rootworm larvae from each
sample were counted, and head capsule width and dry weight measurements were
taken.

Plant Damage

Roots in subplots designated for damage evaluations were dug up, washed, and rated for damage using the node injury scale (NIS) (Oleson et al. 2005). Two sets of subplots were evaluated with the first set being taken on July 9, 2012 when damage to isoline roots had likely peaked based upon the number and size of larvae recovered from the final larval sampling date and soil degree days. The second set of damage evaluations was taken on July 23, 2012. The delay accounted for a potentially slower development of western corn rootworm larvae expected on the transgenic roots (Gray et al. 2007).

Adult Recovery

To ensure collection of emerging adults, emergence traps were placed over the corn on June 19, 2012, which was well before the first predicted western corn rootworm adult emergence of ~700 degree days as calculated by degree day models (Wilde 1971, Levine et al. 1992, Oleson et al. 2005). Emergence traps were placed over either the north or center plant of the three plant subplots. Because of plant spacing issues, only the north or center plant of each subplot was used and the number of adult emergence subplots was doubled to account for this. Emergence trap design was adapted from Hein et al. (1985) with modifications from Pierce and Gray (2007) such that the plant sampled was kept alive. Emergence traps dimensions were 76.2 cm × 45.7 cm and consisted of a wooden frame covered in wire mesh with two holes cut into the center wooden support where the plant is pulled through one hole and tied off using a mesh

sock and cable tie. A funnel was placed into the second hole and a jar fitted opening side

down over the funnel. A metal trim protruded below the wooden frame ~5 cm into the

soil. Emergence traps were situated such that they protruded into the zone of the other

subplot plants as little as possible, however when the center plant was sampled the

south plant had to be destroyed in order to fit the edge of the trap. Adult emergence

traps were kept over the plants until two weeks after the last adult was collected. Both

southern corn rootworm and western corn rootworm were collected two to three times

a week for the duration of the adult sampling period. Southern corn rootworm beetles

were counted and discarded. Total number, head capsule width, sex, and dry weight of

western corn rootworm beetles recovered from each plant were recorded.

Statistical Analysis

To examine larval movement between Bt and isoline plants, we conducted

analysis of variance using PROC MIXED of the SAS statistical package (SAS Institute

2008). For larval recovery, larval dry weight, and plant damage the linear statistical

model contained the main plot effect of treatment, the subplot effect of sample, the

sub-sub plot of plant (center or end plant), and all possible interactions. Data from the

two end plants of each plot were averaged prior to analysis for all factors evaluated,

except beetle emergence, where only one end plant was sampled. Replication ×

treatment was the denominator of F to test treatment. Replication within treatment

and sample date was the denominator for sample time and treatment × sample time.

Plant and all other effects used the residual error for the denominator of F. Although

the tables show the untransformed data, data were square-root (x+0.5) transformed to meet the assumptions of the analysis. For beetle emergence and adult average dry weight, the linear statistical model contained the main plot effect of treatment and the subplot of plant (center or end plant), and the interaction of treatment × plant. Beetle emergence data were further analyzed by estimating the ordinal date for 50% beetle emergence among plants within each treatment and the 95% confidence interval of this point. Data were averaged across replications and beetle sex. The 50% emergence date from the observed cumulative emergence for both years in ordinal dates was calculated using a probit analysis (PROC PROBIT of the SAS statistical package, SAS Institute 2008). There were little to no beetles recovered from the treatment 1 end plant as well as treatments 4 and 5 therefore, calculation of 50% emergence was not possible and these treatments were excluded from the analysis. A generalized linear model (PROC GENMOD) was used to analyze sex ratio of the beetles recovered using a logit link function and a binomial distribution. The sex ratio of the beetles in each treatment were pooled into four categories for this analysis due to low beetle numbers (infest or not infest × Bt or isoline).

Results

Adult Recovery

Overall, the isoline-only treatments had significantly more adults recovered than any other treatment (Table 5, Fig. 7). The mixed treatments in which the Bt plant was infested (Trt 4, 7) had fewer larvae recovered from both plants than the mixed

treatments where an isoline was infested (Trt 3, 8) (Fig. 7). There was no significant difference between adult emergence from the infested isoline plant in treatment 8 and adult emergence from the center Bt plant (Fig. 7). In this treatment the larvae moved from the surrounding infested isoline to the Bt plant in the center and survived. All Bt plants that were infested yielded very few beetles, indicating the Bt proteins are working to control the survival of the rootworms.

The first western corn rootworm beetles emerged on June 29, 2012. Depending on the treatment, time in ordinal days to 50% beetle emergence was between days 185 and 203 (Fig. 8). The few beetles emerged from the Bt plants in treatment 1 and 50% emergence did not differ significantly from the other treatments as suggested seen by the overlapping confidence intervals (Fig. 8). Where there was an infested Bt plant next to an isoline plant (Trt 7), there was a significant delay in the time to 50% emergence in the beetles on that Bt plant from the beetles emerging from either plant in treatment 8 and 3 (infested isoline next to Bt) (Fig. 8).

The sex of the beetles that emerged from the eight treatments differed significantly overall (X^2 =30.00, DF 1, P <0.0001), however the interactions between sex, infestation and seed did not differ significantly (DF 1, X^2=0.72, P > 0.3955). Over 50% of the beetles recovered were female regardless of treatment (Fig. 9). There was no significant difference in the number of males recovered from isoline or Bt plants (P> 0.31), and no difference between females from Bt or isoline (P> 0.32).

There was no significant difference in head capsule widths (HCW) of the adults recovered from all of the treatments (Table 5) (Fig. 10). Overall, the weights of the

adults were similar across treatments (Table 1) (Fig. 11). The weight of the adults

recovered from the Bt plant only treatments was not significantly different from the

weight adults recovered from the isoline only treatments (Fig. 11). On average,

treatment 5 had the smallest beetles, but they were only significantly smaller than

larvae from treatment 4 (Fig. 11).

Damage

Overall, damage was less than 1 on the NIS, even for isoline treatments (Fig. 12).

Treatments in which Bt plants were infested had significantly lower amounts of damage

than treatments in which the isoline plant was infested (Table 5, Fig. 12). In treatments

that had an infested isoline plant, damage was greatest; however, the average damage

was also high in treatment 8 where larvae moved to the Bt plant by the first damage

sample (Fig. 12). In treatment 8, larvae moved early and caused more damage to the Bt

plant than compared to other treatments that contained Bt plants, however this

difference was not significant for the second damage sample as a low amount of

damage were observed on this treatment 8 Bt plant (Fig. 12). In treatment 3, some

larvae did move to the Bt plants but caused an insignificant amount of damage (Fig. 12).

The damage to the infested isoline plants in the isoline only treatments 2 and 6, was

significantly higher than the damage on the infested isoline in treatment 3 where this

plant was surrounded by Bt plants (Fig. 12).

Larval Recovery

The number of larvae recovered from infested isoline plants in mixed treatments did not differ from infested isoline plants in the block refuge control in the all isoline treatments 2 and 6 (Tables 5, 6). Overall, there were no significant differences between the mean number of larvae recovered from the first four larval sample times. By the fifth sample the larvae were probably starting to pupate so there was significantly less recovery (Table 6). The mean number of larvae recovered from infested isoline plants in treatment 8 was significantly higher than mean larvae recovered from the center Bt plant (Table 6). The number of larvae recovered from the center Bt plant in treatment 8 was not significantly different than larvae recovered from non-infested isoline plants in isoline only treatments 2 and 6 (Table 6). In the second (L2) sample (V8 corn stage), almost half as many larvae were recovered from the uninfested Bt plant (21) as surrounding infested isoline plants (49). However there were no significant differences in larval recovery in third-fifth (L3-L5) samples between infested isoline plants and the center Bt plant in treatment 8 (Table 6). Bt only treatments 1 and 5 had consistently low larval recovery throughout the five samples (Table 6). The infested isoline plant in treatment 3 had high numbers of larvae recovered from the plots for the first, second and third, however by third the larvae recovered from the surrounding uninfested Bt plants increased, as some larvae moved (Table 6).

Overall, the isoline-only treatment 2 had the heaviest average larval weight compared to all other treatments, and the larval weight from the all Bt plants in treatment 2 had the lowest average weight (Table 7). There was no significant

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difference in the weight of the larvae recovered from the infested isoline plants and the larvae that had moved to the uninfested Bt plant (Tables 5, 7). In treatment 8, the weight of the larvae recovered from the uninfested center Bt plant was significantly less the weight of the larvae from the surrounding infested isoline plant for the third and fourth samples (Table 7). The weight of the larvae recovered from treatment 4 with the infested Bt plant was significantly smaller than the weight of the larvae recovered from the surrounding uninfested isoline plant (Table 7). The average weight of the larvae recovered from the infested isoline plants was not significantly different regardless of whether they were adjacent to a Bt plant or another isoline plant as in the block refuge control scenario (Table 7).

Head capsule widths from the few larvae recovered from treatment 1 were significantly smaller than head capsule widths of all other beetles recovered from other treatments (Tables 5, 8). The head capsule widths of beetles recovered from uninfested Bt plants in treatment 3 and 8 were not significantly different than head capsule widths from larvae recovered from adjacent infested isoline plants (Table 8). The average head capsule width did not differ between larvae recovered from infested isoline plants in any treatment (Table 8).

Discussion

Overall, given the low adult recovery and low damage to Bt plants, the product performed similar to expectations (Hibbard et al. 2011). The current data show that WCR larvae will move from an infested isoline plant to an uninfested Duracade plant

late in the season. However, a low amount of damage (<0.4 NIS) on average occurred to those Bt plant roots. Although the damage to the uninfested Bt plant in treatment 8 was low, there were still adults recovered from this Bt plant, and the larval recovery data show that an average of 21 larvae moved over to the Bt plant from the isoline plant by the second larval sample time. Even though both plants surrounding the Bt plant in our study in treatment 8 were infested isoline plants, it is unlikely that the larvae moved due to food shortage because damage to these isoline plants did not reach higher than ~0.6 NIS (Hibbard et al. 2004). These larvae were between second and third instar as indicated by the mean head capsule widths (0.43 mm) (Table 6) (Hammack et al. 2003). Larger larvae are potentially more tolerant of the Bt toxins. Binning et al. (2010) found that there was increased larval survival on Cry34/35Ab1 plants with 65% of larvae surviving until adulthood after moving from isoline to Bt plants as early third instar larvae, while only 0.5% of the neonates survived. Zukoff et al. (2012) recovered more adults from the Bt plant when surrounded by two infested isoline plants, and those larvae moved over later (second to third instar) to the Bt plant as indicated by the larval recovery and damage samples. This is further evidence of larvae having greater tolerance to Bt toxins if exposed during a later instar and surviving to adulthood. Meihls et al. (2008) found that their Late-exposure colony, in which a neonate was reared on isoline corn for one week and then Bt corn from then until pupation, had developed resistance after six generations of laboratory selection, however this colony developed resistance more slowly than the constant-exposure colony. The scenario of a Bt plant

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surrounded by isoine plants on each side would only occur 0.2375% of the time in a 5% blended refuge.

Larval movement also occurred from the Bt plants to the isoline plants in treatment 4 and 7. Movement could have been facilitated due to a non-preference behavior of the WCR larvae towards the Bt corn. Clark et al. (2006) found in laboratory studies that some WCR larvae will exhibit a non-preference behavior for Bt plants by only feeding on root hairs and not boring into the root of the Bt plant. Hibbard et al. (2005) found in field experiments that WCR larvae prefer isoline plants to Bt plants, and they will move to a Bt plant only after significant damage is done to the infested isoline plant. Murphy et al. (2010) suggests that the rootworm may leave a Bt root because of non-preference and find an isoline plant just by chance. They hypothesize that this movement from the Bt plant is not necessarily directed towards the isoline plant. Small numbers of larvae were moving from the surrounding Bt to the center isoline during the current study during all of the L1-L5 sample dates (Table 6), and this seed mix scenario will occur 4.5125 percent of the time in a 5% blended refuge. The extent of resistance in these rootworms is unknown. If larvae moved from Bt plants early, then this likely will not select for resistance. Meihls et al. (2008) found that resistance did not develop in their Neonate-exposure colony, in which a neonate was placed on a Bt seedling, but were allowed to immediately crawl off the Bt seedling to isoline corn. However, if larvae are moving off the Bt plant later, then this likely select for resistance.

The sex ratio of WCR beetles was skewed towards females in not only the Duracade treatments, but also in isoline treatments. The reason for this is not clear,

however, Hibbard et al (2011) also detected a female bias (61%) in the adult emergence from the Duracade plants. They did not detect this difference however, in the isoline plants, which showed 51% female recovery. Hibbard et al. (2011) determined that MIR604 had 50% female recovery and eCry3.1ab had 66% female recovery, however the Duracade plants in this study showed an average of 55% female recovery. The reduction in female bias (61% to 55%) found in this study is positive from a management perspective as having unequal sex ratios recovered from Bt plants could result in increased resistance due to increased nonrandom mating (Spencer et al. 2013).

Synchronous emergence of WCR adults from Bt and isoline plants is important for refuge to work properly in reducing the evolution of resistance (Kang and Krupke 2009). Murphy et al. (2010) found that beetles from the isoline plants in the mixed treatment (Bt and isoline) emerged synchronously with the Bt plants and differed from the emergence timing of the beetles recovered from the block refuge. Hibbard et al. (2011) found a 4.6 d delay in the time to 50% emergence between the Duracade and the isoline plants. In our study, the beetles that were recovered from the infested Bt plants showed a 6 d delay in time to 50% emergence to beetles recovered from the uninfested Bt plants in the mixed treatments, and a 7 d delay to the beetles emerging from the isoline plants. These data suggest that when WCR larvae feed on an isoline plant, then move later to a Bt plant (as was found in treatment 8), the emergence dates of those beetles may be synchronized with the beetles emerging from the all isoline plants. If the WCR larvae feed entirely on the Duracade plant, they will emerge up to 7 days later than the former.

Murphy et al. (2010) found that the number of beetles emerging from the isoline plants in the mixed (Bt and isoline) fields was significantly less than the beetles emerging from the 10 and 20% block refuge fields. Our study demonstrated that the susceptible population emerging from the infested isoline plants in the blended refuge scenarios (Bt next to isoline) will have an equal emergence size and fitness compared to the susceptible population emerging from the isoline only plants in our "block refuge" control treatment. The number of larvae recovered from infested, isoline plants, regardless of treatment, was not significantly different from the mixed (isoline/Bt) plant treatments or the "block refuge" isoline-only treatments. The head capsule size as well as the mean larval weight did not differ significantly between any of the infested isoline plants regardless of treatment. The number of adults recovered from the infested isoline plants in the mixed treatments did not differ from the adults recovered from the isoline only treatment (Fig. 12).

A similar study was conducted in 2010 and 2011 by Zukoff et al. (2012) in which movement between isoline and SmartStax plants was documented. This study showed that movement of larvae to the uninfested SmartStax plant from the surrounding infested isoline plants can cause significantly greater amounts of damage to the Bt plant, however this damage was only significant in one of the two years. In the second year of the study, the damage was lower and there were fewer adults collected from the uninfested Bt plant surrounded by infested isoline plants (Zukoff et al. 2012). The results from this Duracade study are similar to the second year of Zukoff et al. (2012), where the larvae caused low amounts of damage to the Bt plant next to isoline plants

later in the season. Although, this field experiment was irrigated and hand watered as often as possible, weather could have played a role in the survival of the rootworms due to the extreme drought (8.6 cm total 5/15-8/17) and consistently high daytime temperatures (avg. 31.6°C) that occurred over much of the corn growing season in 2012 in Boone, Co, Missouri.

Since Syngenta will likely apply for seed mix for this next generation product, an understanding of larval movement between isoline and Bt plants is helpful for regulatory approval. Seed mixes provide the advantage of greater probability of random mating between males and females that emerge from Bt and isoline plants (Kang and Krupke 2009, Murphy et al. 2010, Spencer et al. 2013) which is important for IRM plans to work properly. Our data show that the number of adults produced by isoline plants in the seed mix is comparable to our block refuge control and should provide adequate number of adults. There is an ongoing debate about reduced refuge sizes in light of the recent resistance problems in the field to Cry3Bb1 (Gassmann et al. 2011) and possibly mCry3A (see Chapter 6). The data in the current study suggest that resistance is not likely to develop right away when the larvae move from an infested isoline plant to an uninfested Bt plant later as larger larvae are more tolerant of the Bt toxins (EPA 2002). The low number of WCR larvae that did move from an infested Bt plant to an isoline plant could potentially select for resistance if they survived to adulthood.

Acknowledgements

Table 5. Factors influencing WCR including Analysis of Variance tables for the no. of larvae recovered, larval HCW and dry weight (mg), no. of adults recovered and adult dry weight (g) and HCW and root damage rating recovered from the corn field in 2012.

	Effect	DF	F Value	Pr>F
Adult no.	Trt	7, 28	14.28	<.0001
	plant	1, 32	1.76	0.1946
	Trt*plant	7, 32	2.15	0.0667
Adult wt.	Trt	7, 20	2.88	0.0300
	plant	1, 13	0.49	0.4977
	Trt*plant	6, 13	0.4	0.8669
Adult HCW	Trt	7, 20	1.35	0.2809
	plant	1, 13	1.51	0.2403
	Trt*plant	6, 13	0.29	0.9325
Damage	samptime	1, 32	2.96	0.0948
	Trt	7, 28	19.19	<.0001
	samptime*Trt	7, 32	0.67	0.6975
	plant	1, 59	5.33	0.0245
	samptime*plant	1, 59	0.02	0.8969
	Trt*plant	7, 59	4.11	0.0010
	samptime*Trt*plant	7, 59	0.12	0.9966
Larval no.	samptime	4, 284	5.97	0.0001
	Trt	7, 28	37.41	<.0001
	samptime*Trt	28, 284	2.65	<.0001
	plant	1, 284	0.63	0.4296
	samptime*plant	4, 284	0.34	0.8516
	Trt*plant	7, 284	16.36	<.0001
	samptime*Trt*plant	28, 284	1.89	0.0053
Larval HCW	samptime	4, 95	51.03	<.0001
	Trt	7, 26	6.93	0.0001
	samptime*Trt	27, 95	1.24	0.2238
	plant	1, 88	7.18	0.0088
	samptime*plant	4, 88	1.55	0.1955
	Trt*plant	7, 88	3.74	0.0014
	samptime*Trt*plant	23, 88	1.6	0.0623
Larval weight	samptime	4, 95	30.64	<.0001
	Trt	7, 26	5.59	0.0005
	samptime*Trt	27, 95	0.97	0.5149
	plant	1, 86	0.09	0.7646
	samptime*plant	4, 86	2.68	0.0369
	Trt*plant	7, 86	4.05	0.0007
	samptime*Trt*plant	23, 86	1.87	0.0204

Table 6. Western corn rootworm larvae (means±SE) recovered from each treatment over five sample dates from the corn field in 2012.

				Corn developmental stage at recovery						
Plant	Trtmt	Seed	Infest	V6	V8	V11	V12	VT		mean
plant	trt	seed	infestion	L1	L2	L3	L4	L5	LSD	
center	1	Bt	infested	0.00	0.8±0.80	0.2±0.20	0.8±0.37	2.2±1.24	1.45	0.64
north	1	Bt	noinf	0.00	0.1±0.10	0.2±0.20	0.1±0.10	0.00	1.45	0.08
center	2	iso	infested	44±13.69	21.6±8.44	20.2±10.17	26.2±6.28	4.0±1.00	1.45	23.20
north	2	iso	noinf	4.2±1.71	9.4±2.35	11.7±5.03	18.8±3.75	4.0±1.52	1.45	9.62
center	3	iso	infested	20.8±7.62	24.8±10.58	40±18.59	8.4±2.71	10.2±3.77	1.45	20.84
north	3	Bt	noinf	2.4±0.66	5.2±2.70	8.3±2.33	3.3±2.00	3.0±1.64	1.45	4.44
center	4	Bt	infested	3±1.52	1.2±0.97	2.8±1.85	1.2±0.73	1.4±0.75	1.45	1.92
north	4	iso	noinf	0.8±0.58	2.9±0.73	2.6±1.12	3±1.58	1.4±0.48	1.45	2.14
center	5	Bt	noinf	0.00	1.4±1.40	0.2±0.20	0.4±0.24	0.00	1.45	0.40
north	5	Bt	infested	0.5±0.39	0.1±0.10	0.2±0.12	0.00	2.2±1.96	1.45	0.60
center	6	iso	noinf	21.6±6.88	13.8±5.39	17.8±6.12	9.2±1.77	8.6±2.42	1.45	14.20
north	6	iso	infested	38.2±11.62	37.1±8.77	21.2±8.27	23.6±5.00	7.2±1.67	1.45	25.46
center	7	iso	noinf	2.2±1.02	4±2.14	6.8±6.05	4.8±1.66	4.25±2.72	1.45	4.41
north	7	Bt	infested	2.1±1.25	1.6±0.48	1.9±0.81	1.5±0.76	2.25±1.36	1.45	1.87
center	8	Bt	noinf	1.8±1.11	21.8±10.89	9.2±3.69	10.4±2.96	10.25±5.54	1.45	10.69
north	8	iso	infested	39.5±17.8	49.1±10.21	23.9±3.16	18±4.29	10.7±2.39	1.45	28.24
LSD				1.50	1.50	1.50	1.50	1.50		0.78
mean				11.3	12.18	10.45	8.36875	4.478125	0.36	

Table 7. Western corn rootworm larvae dry weight (mg) (means±SE) recovered from each treatment over five sample dates from the corn field in 2012.

Weight				Corn developmental stage at recovery						
Plant	Trtmt	Seed	Infest	V6	V8	V11	V12	VT		mean
plant	trt	seed	infestion	L1	L2	L3	L4	L5	LSD	
center	1	Bt	infested	.	0	0.03	0.47±0.32	0.24±0.15	0.36	0.24
north	1	Bt	noinf	.	0	0.12	0.22	.	0.44	0.11
center	2	iso	infested	0.14±0.02	0.49±0.07	0.59±0.14	0.88±0.04	0.74±0.19	0.20	0.57
north	2	iso	noinf	0.25±0.04	0.95±0.08	1.10±0.18	1.25±0.1	1.26±0.19	0.20	0.96
center	3	iso	infested	0.16±0.04	0.52±0.09	0.43±0.09	0.84±0.19	0.75±0.09	0.20	0.54
north	3	Bt	noinf	0.10±0.06	0.53±0.17	0.54±0.04	0.94±0.1	0.80±0.34	0.21	0.58
center	4	Bt	infested	0.64±0.55	0.26±0.26	0.62±0.24	0.24±0.09	0.83±0.16	0.25	0.52
north	4	iso	noinf	0.05±0.05	0.86±0.15	0.78±0.05	1.25±0.25	1.03±0.25	0.23	0.79
center	5	Bt	noinf	.	1.57	0.30	0.18±0.1	.	0.38	0.68
north	5	Bt	infested	0	0	0.45±0.45	.	0.98±0.08	0.35	0.35
center	6	iso	noinf	0.19±0.05	0.72±0.21	0.64±0.11	1.01±0.09	0.89±0.06	0.20	0.69
north	6	iso	infested	0.13±0.02	0.70±0.06	0.78±0.11	1.08±0.13	0.74±0.07	0.20	0.69
center	7	iso	noinf	0.09±0.04	0.69±0.39	1.30±0.47	0.77±0.05	0.88±0.18	0.24	0.75
north	7	Bt	infested	0.04±0.01	0.22±0.12	0.30±0.07	1.37±0.7	1.15±0.31	0.24	0.62
center	8	Bt	noinf	0.10±0.02	0.38±0.04	0.64±0.19	0.81±0.12	0.60±0.06	0.23	0.51
north	8	iso	infested	0.18±0.03	0.51±0.05	0.87±0.07	1.12±0.15	0.75±0.09	0.20	0.69
LSD				0.23	0.26	0.23	0.21	0.23		0.10
mean				0.15	0.53	0.59	0.83	0.83	0.08	

Table 8. Western corn rootworm larvae head capsule widths (mm) (means±SE) recovered from each treatment over five sample dates from the corn field in 2012.

HCW				Corn developmental stage at recovery						
Plant	Trtmt	Seed	Infest	V6	V8	V11	V12	VT		mean
plant	trt	seed	infestion	L1	L2	L3	L4	L5	LSD	
center	1	Bt	infested	.	0.25	0.35	0.43±0.07	0.38±0.08	0.07	0.35
north	1	Bt	noinf	.	0.15	0.30	0.30	.	0.09	0.25
center	2	iso	infested	0.33±0.01	0.41±0.02	0.41±0.03	0.46±0.01	0.43±0.03	0.04	0.41
north	2	iso	noinf	0.36±0.03	0.46±0.01	0.45±0.01	0.46±0.01	0.45±0.01	0.04	0.44
center	3	iso	infested	0.30±0.01	0.39±0.02	0.41±0.03	0.42±0.03	0.46±0.01	0.04	0.40
north	3	Bt	noinf	0.27±0.01	0.44±0.02	0.43±0.02	0.48±0.01	0.45±0.02	0.04	0.41
center	4	Bt	infested	0.33±0.09	0.36±0.16	0.44±0.02	0.37±0.04	0.45±0.03	0.04	0.39
north	4	iso	noinf	0.23±0.03	0.47±0.03	0.43±0.02	0.50±0.01	0.4±0.05	0.04	0.41
center	5	Bt	noinf	.	0.47	0.45	0.38±0.07	.	0.08	0.43
north	5	Bt	infested	0.17±0.02	0.20	0.35±0.15	.	0.48±0.02	0.07	0.43
center	6	iso	noinf	0.29±0.03	0.42±0.04	0.45±0.02	0.46±0.02	0.47±0.02	0.04	0.42
north	6	iso	infested	0.30±0.01	0.44±0.02	0.45±0.01	0.46±0.01	0.46±0.01	0.04	0.42
center	7	iso	noinf	0.26±0.03	0.38±0.05	0.49±0.05	0.43±0.02	0.45±0.03	0.04	0.40
north	7	Bt	infested	0.23±0.01	0.36±0.02	0.39±0.03	0.50±0.02	0.42±0.04	0.04	0.38
center	8	Bt	noinf	0.26±0.01	0.43±0.02	0.43±0.03	0.49±0.02	0.46±0.02	0.04	0.41
north	8	iso	infested	0.30±0	0.39±0.01	0.46±0.02	0.45±0.01	0.44±0.01	0.04	0.41
LSD				0.05	0.05	0.05	0.04	0.04		0.02
mean				0.28	0.35	0.42	0.44	0.44	0.02	
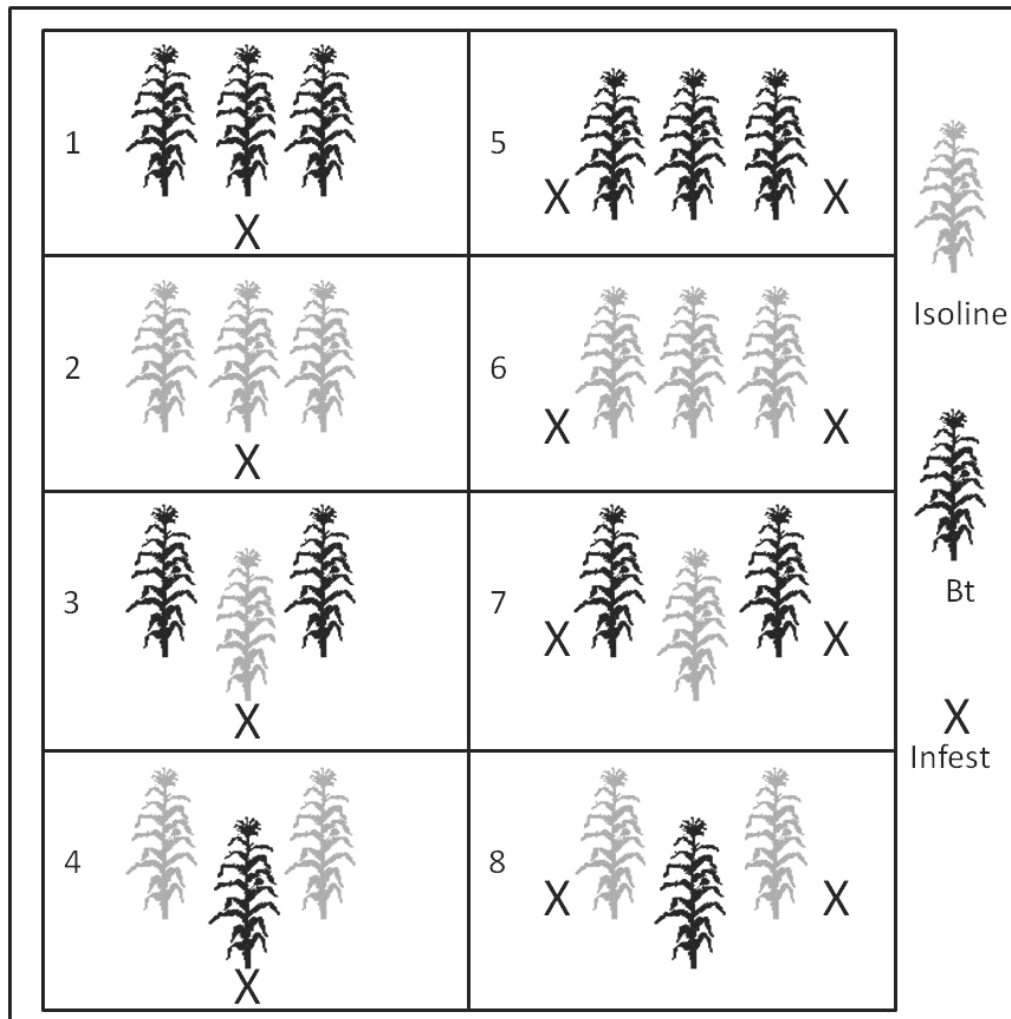
Figure 6. Planting and infestation combinations of the eight treatments. In Treatments 1-4 the center plant was infested with western corn rootworm eggs and in Treatments 5-8 the end plants were infested. In Treatment 3 and 4 the middle seed was planted slightly off center so the larvae, once hatched, would have to travel through the roots to reach another plant. Figure adapted from Zukoff et al. 2012.
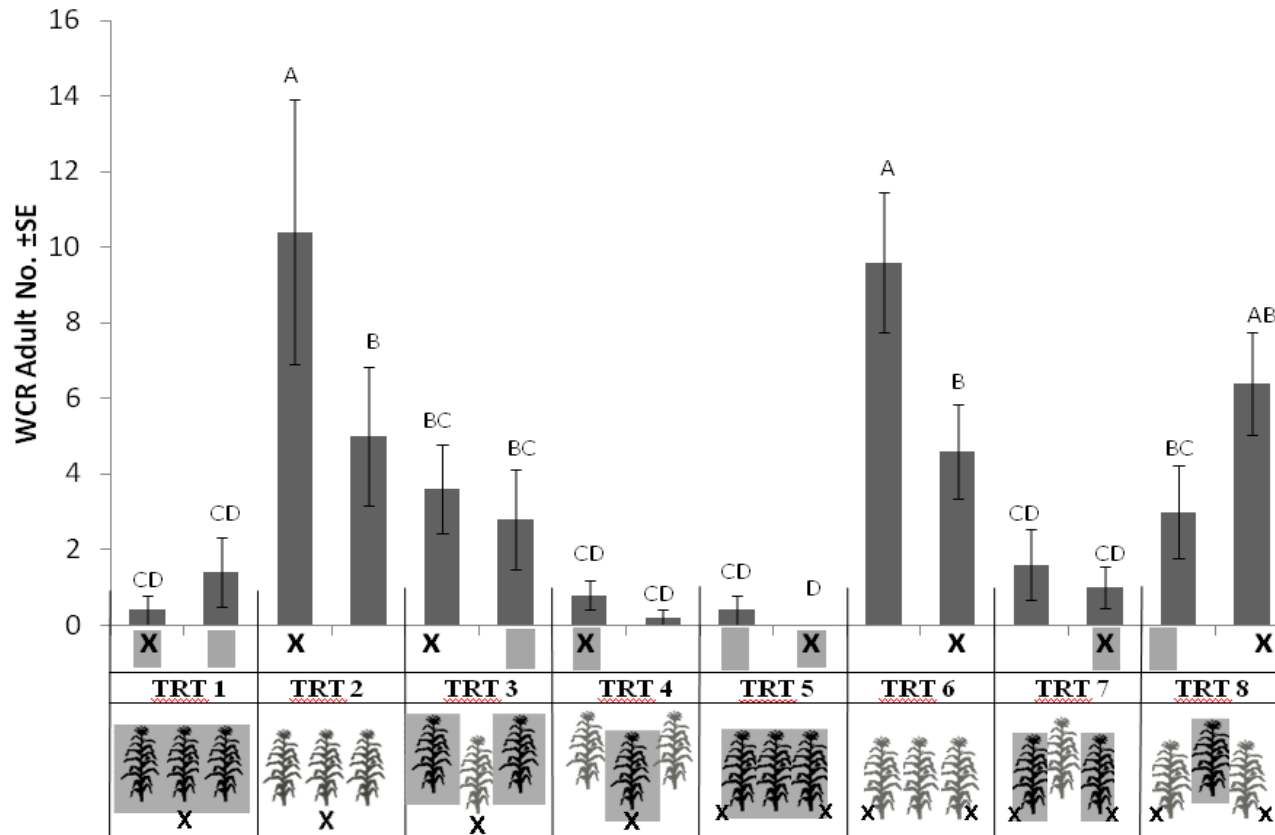
Figure 7. Mean±SE number of adult western corn rootworm recovered in the emergence traps in eight treatments from the corn field in 2012. The numbers indicate the treatment groups. The two end plants in each treatment were combined. The gray boxes with black corn indicate Duracade plants, the gray corn symbols indicate isoline plants and the X signifies the infested plants. Different letters indicate significant difference ($P \leq 0.05$).
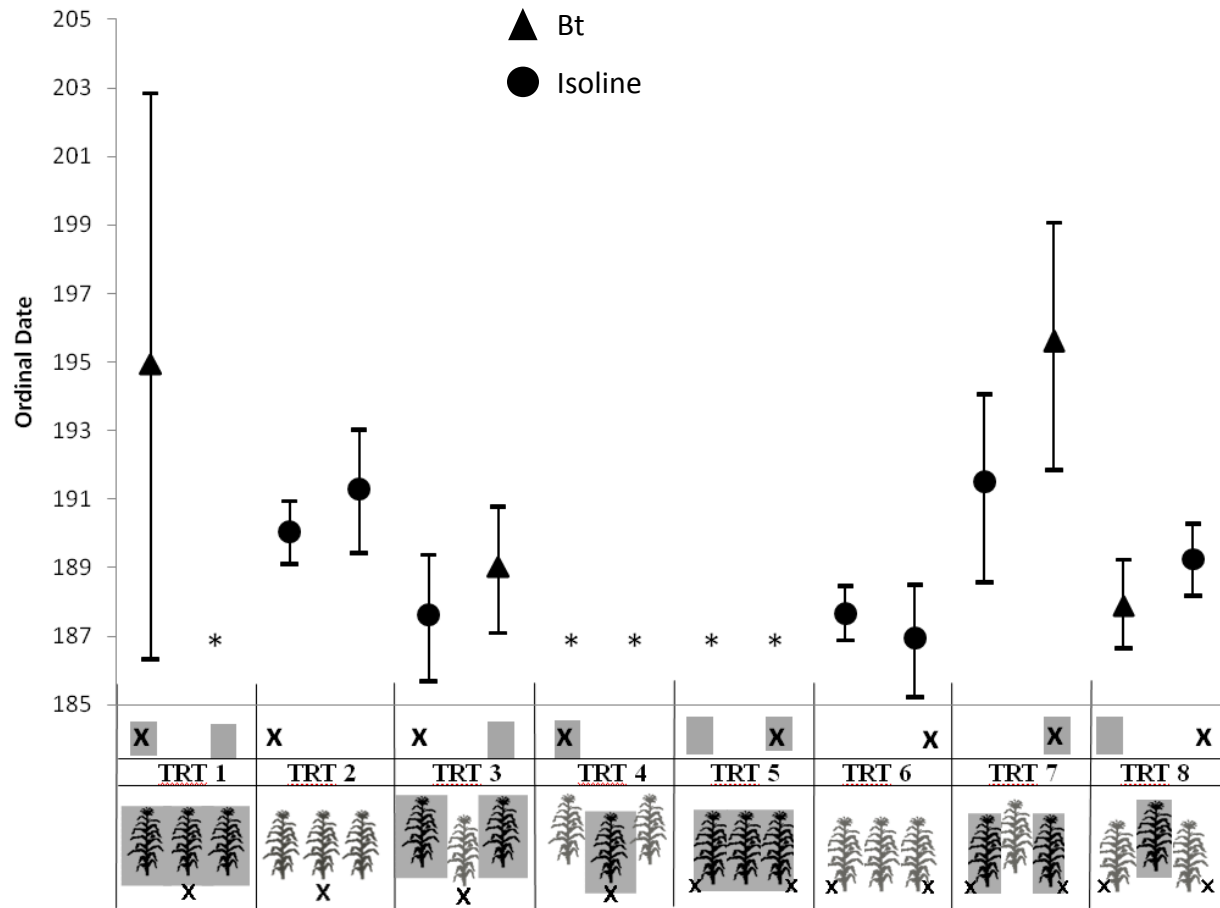
Figure 8. Ordinal dates for 50% emergence of adult western corn rootworm (*Diabrotica virgifera virgifera*) from the Bradford Farm, Columbia, MO corn field in 2012. * indicates treatments that had too few beetles to be included in the analyses.
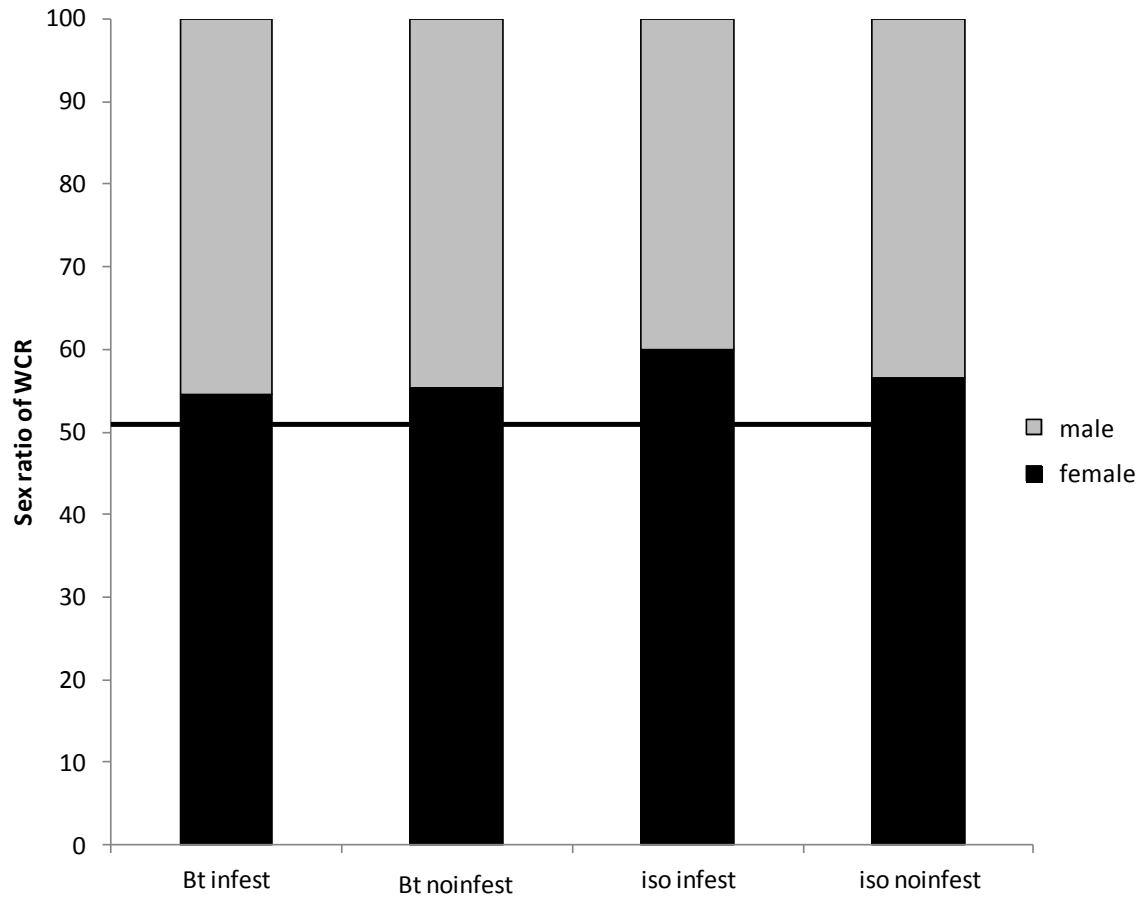
Figure 9. Sex ratio of WCR beetles (*Diabrotica virgifera virgifera*) recovered from the emergence traps from the corn field at Bradford Farm, Columbia, MO. Beetle numbers from the eight treatments were pooled due to low numbers and grouped by seed (Bt or isoline) and infestation (infested or not).
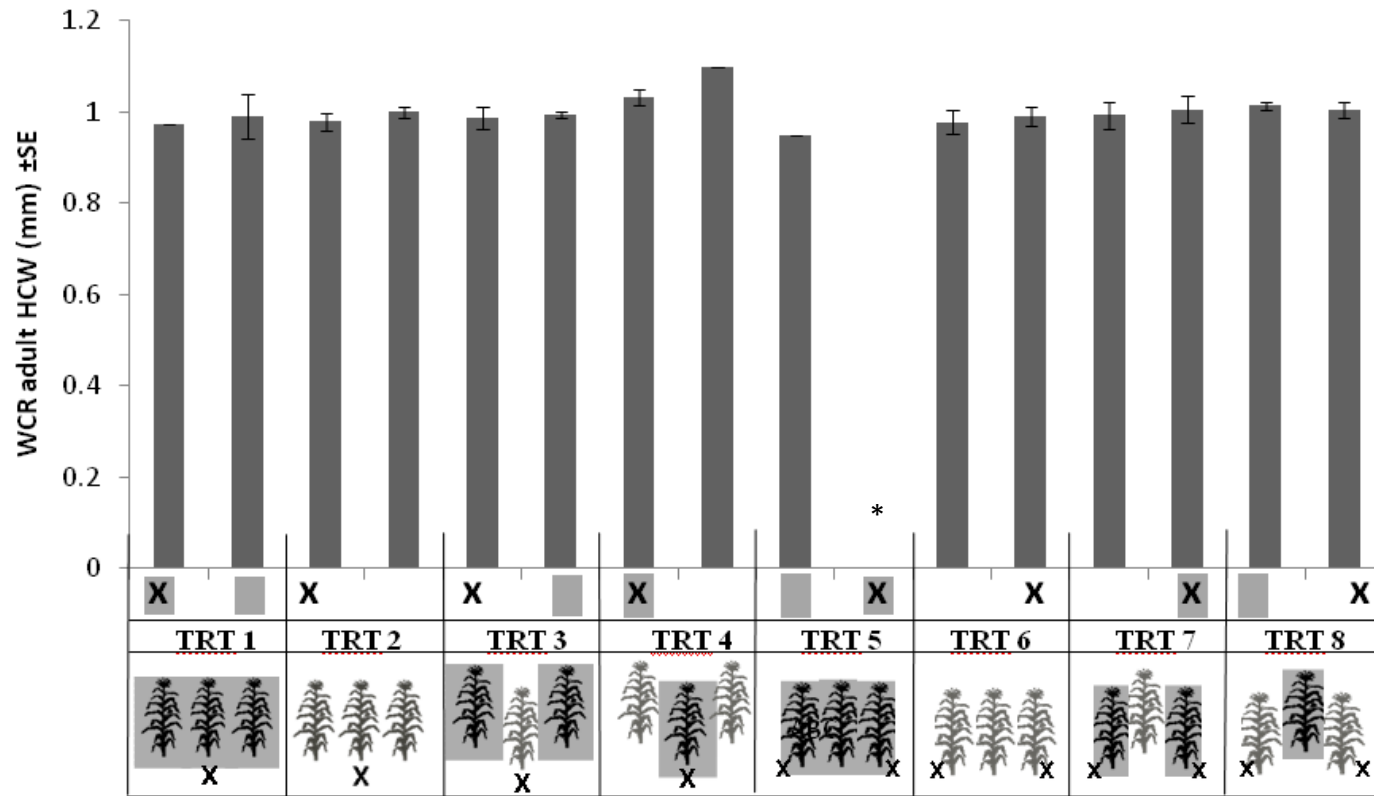
Figure 10. Mean±SE head capsule width (HCW) of adult western corn rootworm recovered in the emergence traps in eight treatments from the corn field 2012. The gray boxes with black corn indicate Duracade plants, the gray corn symbols indicate isoline plants and the X signifies the infested plants. There was no significant difference in HCW of the adults recovered. * No adults recovered.
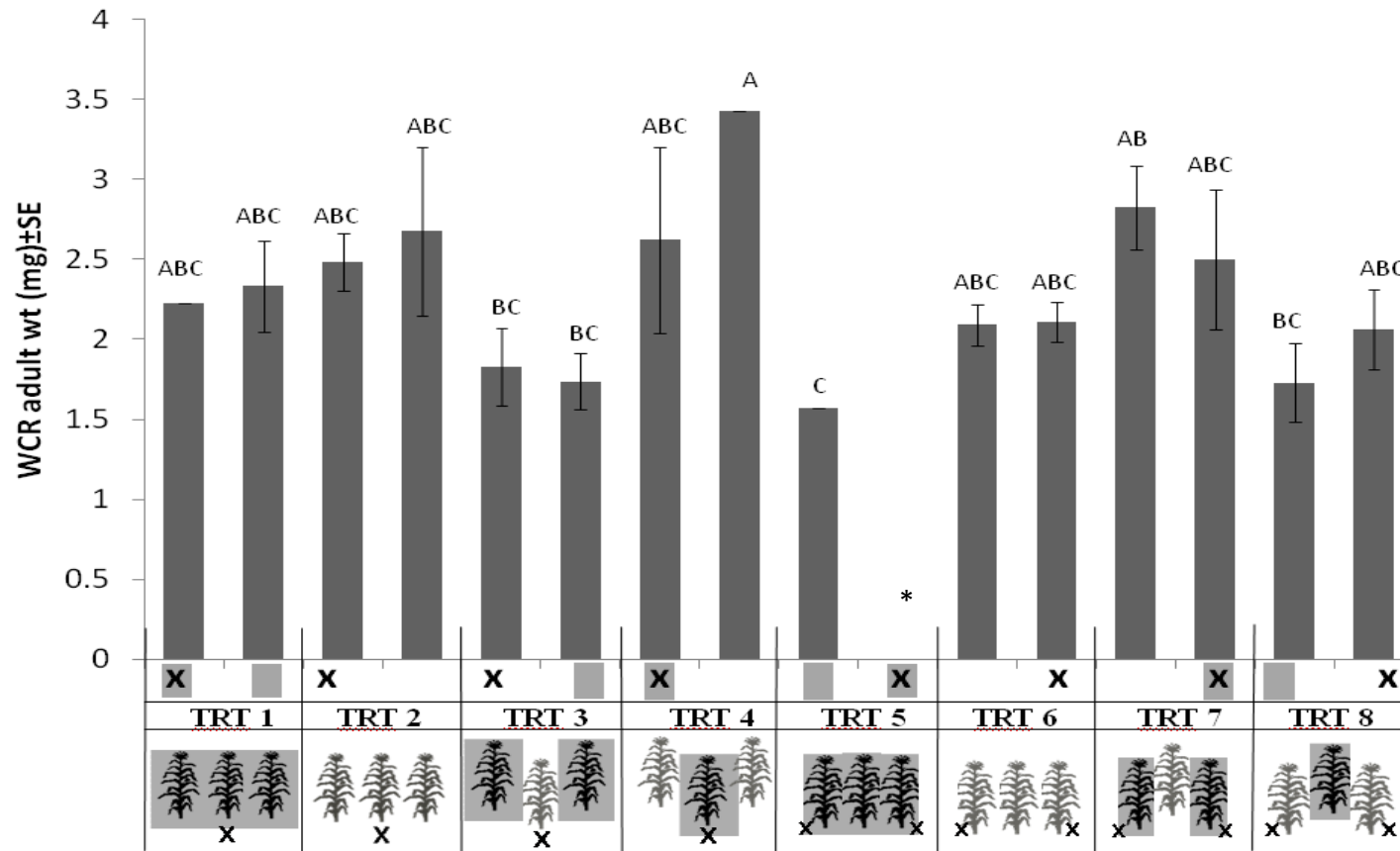
Figure 11. Mean±SE dry weight of adult western corn rootworm recovered in emergence traps in eight treatments from the corn field in 2012. The numbers indicate the treatment groups. Data for the two end plants in each treatment were combined. The gray boxes with black corn indicate Duracade plants, the gray corn symbols indicate isoline plants and the X signifies the infested plants. Different letters indicate a significant difference (*P* ≤0.05). * No adults recovered.
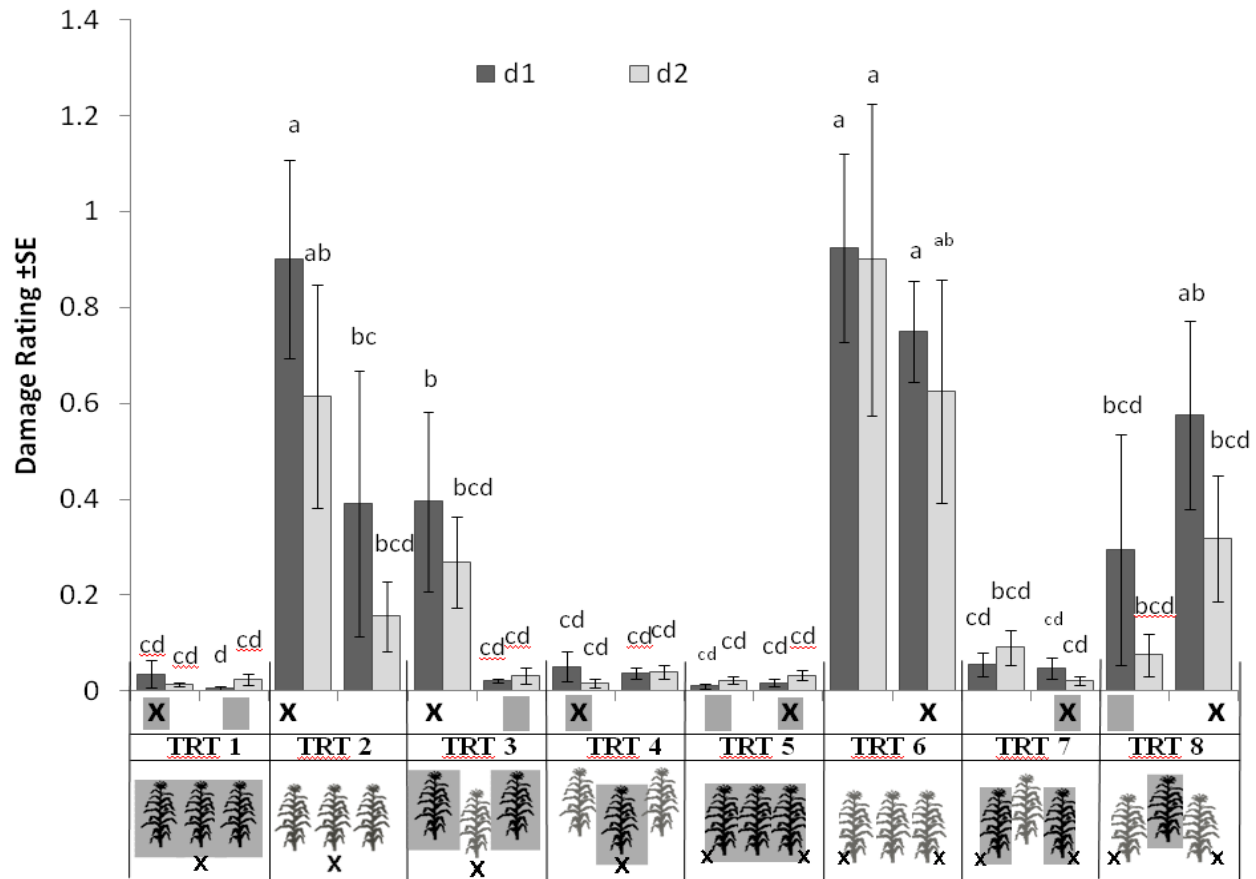
Figure 12. Mean±SE damage rating from two sampling periods in eight treatments of Bt and isoline corn plants from the corn field in 2012. The number indicates treatment group. The two end plants in each treatment were combined. The gray boxes with black corn indicate Duracade plants, the gray corn symbols indicate isoline plants and the X signifies the infested plants. The same letters indicate no significant difference (*P* ≤0.05).

CHAPTER IV: HOST RECOGNITION RESPONSES OF WESTERN CORN ROOTWORM LARVAE TO BT CORN

Introduction

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is considered to be the most important insect pest of corn (*Zea mays* L.) in major corn producing regions of the U.S. (Stamm et al. 1985, Krysan et al. 1986) with crop losses and control costs estimated to be over $2 billion annually in the U.S. alone (Mitchell 2011). WCR larvae are subterranean and specialize on corn roots. WCR larvae will feed on most grasses (Family Poaceae), but can only complete their development on a select few species other than corn (Branson and Ortman 1970, Clark and Hibbard 2004). Larvae are the most economically damaging stage of WCR due to intense feeding on the root system, which can cause major difficulty with nutrient and water uptake in the plants (Kahler et al. 1985, Sutter et al. 1990). This damage can weaken the plant base and cause the plants to fall over or "lodge", especially during periods of heavy winds and rain, which make harvesting with a combine very difficult.

WCR eggs are laid in soil near the base of the corn plant except where rotation-resistant varieties have evolved that have lost their fidelity to corn and lay their eggs in soybeans and other crops (Onstad et al. 2003, Gray et al. 2009). The larvae use CO_2, which is given off by all plants, as a long range attractant as they move through the soil in search of host roots (Branson 1982, Strnad et al. 1986, Hibbard and Bjostad 1988, Bernklau and Bjostad 1998, Miller et al. 2006). Another important volatile is ethylene,

which is a phytohormone in corn that the larvae use to locate hosts roots (Robert et al. 2012). WCR larvae are also attracted to (*E*)-β-caryophyllene, which is an induced plant volatile given off when WCR larvae feed on the roots of certain corn varieties. Recently, Robert et al. (2012) discovered that both of these volatiles are used by the larvae to evaluate the health of the plant from a distance. Although older larvae can survive starvation for up to 96 hours, neonate larvae need to locate host roots within 12-36 hours or risk being too weak to burrow into the root (Strnad and Bergman 1987a).

Once the WCR larvae find the roots, contact cues are picked up by the maxillary palps to aid in feeding decisions (Branson and Ortman 1969). Feeding stimulants used by the WCR larvae to identify a host have been identified as a combination of simple sugars, 30:4:4 mg/ml glucose:fructose:sucrose, and one of the free fatty acids in germinating corn roots, oleic acid or linoleic acid (Bernklau and Bjostad 2008). Interestingly, individual components by themselves did not elicit a major feeding response by the WCR larvae, but together, they did (Bernklau and Bjostad 2008).

Larvae of the WCR have a set of behaviors that help the larvae locate food patches as well as stay within food patches. When WCR larvae are exposed to a substrate that is not recognized as a host and then are removed, they exhibit a "ranging" behavior, where the larvae crawl in a relatively straight direction and move quickly (Strnad and Dunn 1990). Until the larvae encounter host volatiles, they will continue searching in this manner. In contrast, when WCR larvae are exposed to a host root and then are removed, they exhibit a "localized searching" behavior. This behavior involves a restricted area of search with greater number of turns and a decrease in

speed (Strnad and Dunn 1990, Bell 1991). Throughout their development, WCR larvae move to higher quality, younger root whorls (Apple and Patel 1963, Strnad and Bergman 1987b), and this localized searching behavior likely helps the larvae not stray too far from the root while moving around. These behaviors are important for larval survival and contribute to the highly successful nature of this pest (Strnad and Dunn 1990).

In behavioral bioassays Strnad and Dunn (1990) analyzed the paths that the WCR larvae took after exposure to germinated roots of corn and other grasses. They found that after being exposed to corn and wheat roots, the rootworms initiated localized search. The WCR larvae exposed to giant fox tail and oat (*Avena sativa* L.) seedling roots, both non-hosts of WCR, showed in part localized search by having a reduced area of search and reduced velocity, however, they did not show any differences in the number of turns and path crossing. Although the rootworm larvae will feed briefly on the oats, they will abandon them due to a feeding deterrent (Branson and Ortman 1969). Bernklau et al. (2009) found that WCR larvae will initiate localized search when exposed to root extracts, corn root pieces and corn root juice.

Transgenic corn lines with genes from *Bacillus thuringiensis* (Bt) with resistance to WCR feeding are commonly used for rootworm management in the U.S. These products range from single event hybrids to a pyramided hybrids that have two or more Bt genes targeting rootworms. Current commercially available Bt hybrids targeting the WCR produce one or more of the following proteins mCry3A, Cry3Bb1 or Cry34/35Ab1. SmartStax® is a stacked corn hybrid that is a collaboration between Monsanto Company and Dow AgroSciences, which includes two pyramided rootworm genes, Cry3Bb1 and

Cry34/35Ab1 as well as three Bt toxins targeted towards above ground pests and herbicides. Syngenta's next generation product, Agrisure ® Duracade, which includes mCry3A + eCry3.1Ab, is expected to be commercially available in 2014 pending regulatory approval from the USDA. This product has already received FDA and EPA approval.

WCR host recognition behavior is unknown for these transgenic genes and the recent discovery of populations of WCR resistant to Cry3Bb1 Bt corn in the field (Gassmann et al. 2011) raises concerns about the rootworm-transgenic corn interactions. The objective of this study was to investigate how mCry3A, Cry3Bb1 and Cry34/35Ab1 influence the host recognition behavior of neonate WCR larvae.

Methods

The study was conducted at the USDA-ARS Plant Genetics Research Unit on the University of Missouri-Columbia campus in 2010 and 2011. To assess the host recognition behavior of WCR neonates on different varieties of corn roots, we conducted two sets of bioassays. The first set of bioassays consisted of a randomized complete block with nine treatments with WCR larvae susceptible to all Bt corn types on one of seven types of corn, oat (non-host living plant control) or filter paper (control) with 20 replicates per treatment. The seven corn types used included MIR604 (mCry3A), DAS59122-7 (Cry34/35Ab1), MON88017 (Cry3Bb1), SmartStax (Cry3Bb1+Cry34/35Ab1) and their corresponding isolines.

Insects

WCR eggs were obtained from non-diapausing (Branson 1976) colonies maintained in our laboratory. The egg type used was from an unselected WCR line (Janesville control – see Meihls et al. 2012). WCR eggs were placed in 15 cm × 10 cm oval containers (708 ml, The Glad Products Company, Oakland, CA) and filled approximately 4 cm deep with a growth medium of 2:1 autoclaved soil and ProMix™ (Premier Horticulture Inc.). The eggs were incubated in the soil at 25°C for approx. two weeks before hatching. Unfed neonate larvae used in the bioassays were used less than 24 hours after hatching.

Plant Material

All of the corn used was soaked in a 10% bleach solution for 10 minutes, rinsed well and allowed to dry completely prior to germination. The corn was then soaked in water at room temperature for 8 hours. After soaking, corn kernels were placed onto a saturated paper towel in closed oval containers and placed in a growth chamber at 25°C to germinate. Oats were treated with a soapy water solution, rinsed well and placed on a saturated paper towel in oval containers for germination in the growth chamber. Upon germination, all plants were kept moist on clean, saturated filter paper in closed oval containers. Corn seedlings were used in bioassays when they reached 3-4 days old; oats were used at 4-5 days old. The roots used in the assays were approximately between 1.5 and 2 inches in length.

Gene checks were performed on MON88017 and SmartStax roots at the end of the study using QuickStix test strips (EnviroLogix, Portland, ME.

Bioassays

Assays used in this study were modified from Strnad and Dunn (1990). During the bioassays, a single, clean seedling was placed on moistened filter paper in a petri dish and one neonate larva was placed on the root (or on the filter paper for the control) using a moistened camel's-hair paintbrush. After exposure to the root for 5 min, the larva was transferred to the center of a specially designed 12.5 cm arena on lightly moistened filter paper and its host-searching behavior was recorded for five minutes using the EthoVision system (Version 3.1, Noldus Information Technology, The Netherlands). The bioassay was terminated early if any larva exited the arena during the 5 min trial period. No root was reused in the bioassays. Each bioassay resulted in one track file in the EthoVision program.

EthoVision Protocol

The EthoVision arena comprised of a moist 125 mm filter paper circle (Fisher Scientific Pittsburgh, PA), replaced between bioassays, and was placed on a clean glass plate. This was enclosed in a clear acrylic box (20×20×18 cm) mounted under the EthoVision system video camera (Panasonic wv BP334) positioned 0.64 cm above the box with a 15-W fluorescent light located on top for even lighting. For optimum viewing of larvae with the EthoVision system, the tracking settings were set to the following specifications: detection method, subtraction; processing settings, only detect objects

that are darker than background; scan window of 50 pixels set to search the complete

arena; minimum object size, one pixel; maximum object size, 20 pixels; sample rate,

5.994 samples/sec. Recording began after the larvae were placed in the arena and the

door to the arena closed. The recording continued for 5 min or until the larva left the

filter paper. To account for any changes in the settings due to replacing the filter paper

between bioassays, the detection variables were updated before the start of each trial.

Parameters measured by the EthoVision system during bioassays included: total

distance moved (the distance traveled by the center of gravity of the larva), maximum

distance from the origin (the farthest distance traveled by the center of gravity of the

larva from the point of origin), mean velocity (cm/s), mean turn angle (the change in

direction of movement between two samples), and mean meander (the change in

direction of movement of an object relative to the distance it moves). To mitigate image

noise and larval body wobbles being recorded as true movement, the following filters

and settings were used when calculating the above parameters: total distance moved,

downsize filter (1/25) and minimum distance moved (0.2 cm); maximum distance from

origin, downsize filter (1/25); mean velocity, downsize filter (1/25); mean turn angle,

absolute setting and downsize filter (1/25); mean meander, absolute setting and

downsize filter (1/25). Limited larval movement coupled with the above filters

sometimes resulted in no value being calculated for a specific parameter. For trials that

did not last the full five minutes as a result of larvae leaving the arena during their

search, total distance traveled was adjusted to reflect the distance the larvae would

have traveled during the five minute period using their average velocity as calculated by the EthoVision software.

Statistical Analysis

An ANOVA was used for these data analyses and was calculated by using the PROC MIXED of the SAS statistical package (SAS Institute 2008). For the mean meander, total distance moved, mean turn angle, maximum distance from origin and the velocity the linear statistical model contained the main plot effect of treatment. Data were transformed by square root (x+0.5) to meet the assumptions of the analysis. Both of the experiments were run as a randomized complete block.

Results

For all parameters that were measured, the two negative controls (moist filter paper and germinated oat seedlings) were significantly different than all corn treatments (Table 9, Fig. 13). The larvae that were exposed to the controls had significantly longer paths and traveled farther from the distance from the origin than the larvae exposed to corn plants including the Bt plants (Figs. 13a, b). The larvae exposed to the negative controls traveled significantly faster, turned less and crossed their paths less than the larvae exposed to the corn plants (Figs. 13c,d,e).

Discussion

There were no dramatic differences between the localized search responses of WCR larvae to any of the corn lines tested, however the rootworm larvae consistently

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demonstrated a ranging behavior after contact with the filter paper and oats, indicating

that they did not recognize the controls as hosts. This was expected since oats had

similar results before (Strnad and Dunn 1990) and may contain a feeding deterrent

(Branson and Ortman 1969). Contact cues associated with the roots are the driving

factor of host recognition (Branson and Ortman 1969, Strnad and Dunn 1990), and this

study demonstrates that each corn type, the stack as well as isoline, contains sufficient

contact cues to elicit a localized search response by Cry3Bb1 susceptible larvae when

the larvae are removed from the roots. Apparently, the toxins present in the transgenic

roots did not turn the plants into non-hosts from the perspective of this assay despite

what may have happened in other assays such as Clark et al. (2006).

Higgins et al. (2009) conducted assays that were somewhat similar to the current

experiment, except that in their experiment they exposed the insects to artificial diet

(modified after Pleau et al. (2002) with and without Cry34/35Ab1 proteins. They

concluded that Cry34/35Ab1 was perceived as a poor host for WCR larvae. However, the

factors responsible for host recognition require specific extraction techniques if they are

to be separated from corn (Bernklau et al. 2009), and these factors are likely not present

in artificial diet. In addition, Cry proteins are tied up in plant cells under normal

circumstances and not directly available to searching larvae as was done by Higgins et al.

(2009). In the current studies, all transgenic products were only available to the neonate

insect in plants, and all corn lines were recognized as suitable hosts.

Acknowledgements

Table 9. Effect of treatment on each parameter measured of the movement of the western corn rootworm during Strnad assays from experiment A using susceptible insects and experiment B using both susceptible and resistant insects.

Analysis		df	f	P
Distance Moved	treatment	4, 149	9.80	<0.0001
Mean Velocity	treatment	8, 149	19.16	<0.0001
Mean Turn Angle	treatment	8, 147	41.56	<0.0001
Mean Meander	treatment	8, 147	36.91	<0.0001
Maximum Distance	treatment	8, 147	22.29	<0.0001
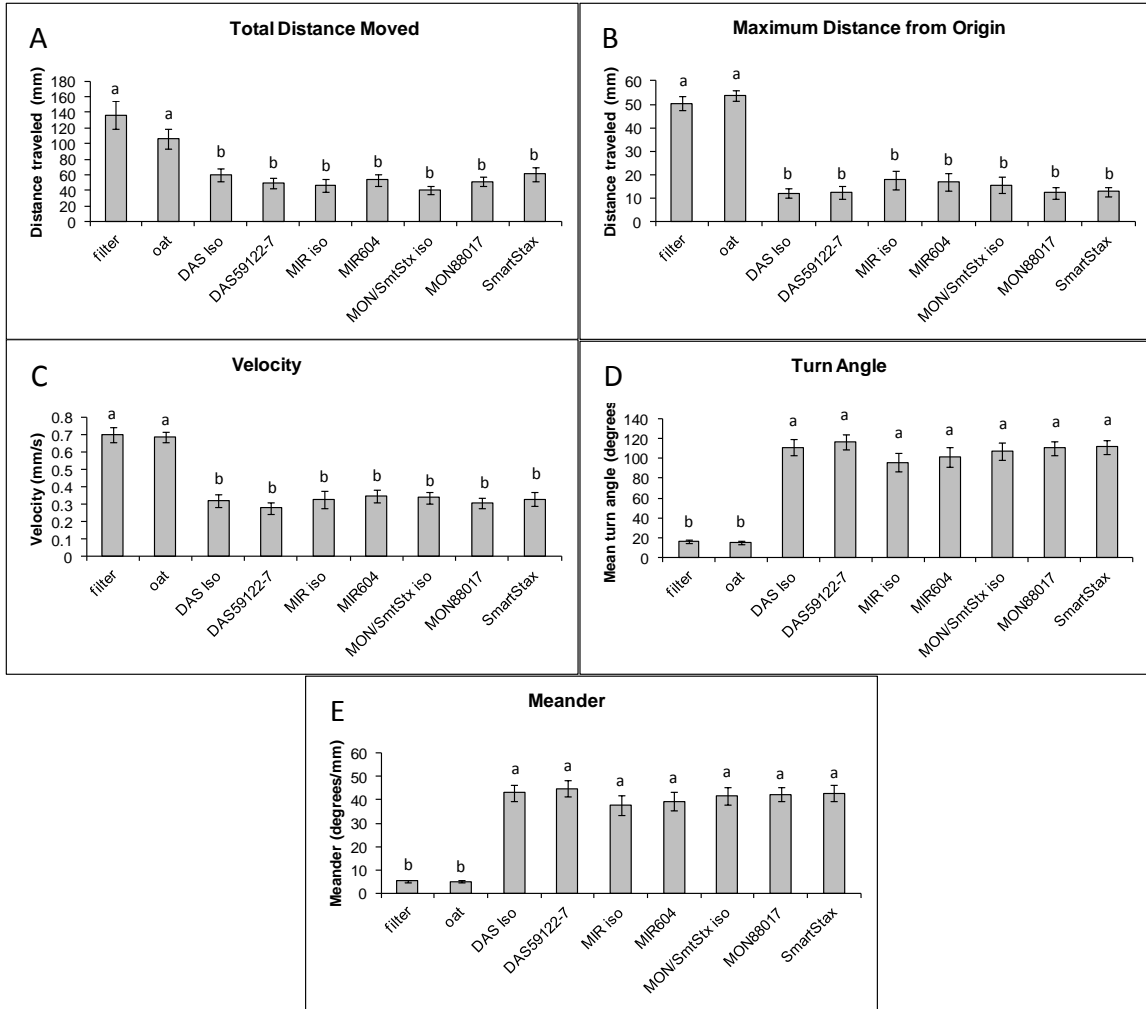
Figure 13. The total distance moved, maximum distance from origin, velocity, turn angle and meander of the western corn rootworm larvae in five minutes after exposure to a different plant seedlings or filter paper for experiment A. Letters indicate significant differences between corn types (p≤0.05). Analysis was done with square root transformed data

CHAPTER V: EVALUATION OF POTENTIAL CROSS RESISTANCE BETWEEN FIELD RESISTANT AND SUSCEPTIBLE POPULATIONS OF THE WESTERN CORN ROOTWORM (*DIABROTICA VIRGIFERA VIRGIFERA*) ON MCRY3A, CRY3BB1 AND CRY34/35AB1 CORN IN LABORATORY AND GREENHOUSE ASSAYS

Introduction

The adoption of Integrated Pest Management (IPM) (Stern et al. 1959) in the United States was facilitated by a common goal of reducing pesticide exposure to the environment and humans by using an integrated approach to control pests (USDA 1993). This combined approach uses knowledge of the biology of the pest as well as knowledge of all control methods to create a plan that is both economically sound and minimizes the hazardous substance exposure to the environment. Insect Resistance Management (IRM) plans are used to maintain insects susceptible to management tactics and to further the longevity of the management tactic for future use (Bates et al. 2005). Hybrid corn incorporating genes from *Bacillus thuringiensis* (Bt) were introduced to control target pests and have been widely accepted because they were highly effective, brought value to the grower, reduced the need for pesticides, and limited harm to non-target species and the environment. The EPA requires IRM plans with each Bt hybrid registered for commercial sale and this involves planting a certain percentage of the Bt field or nearby fields with refuge or non-Bt plants (EPA 1998). The theory behind the refuge use is based on a high-dose refuge strategy where resistance alleles are assumed to be recessive, and the rare resistant insect that survives the Bt will mate

with those more abundant susceptible insects from the refuge to create susceptible

offspring and thereby inhibit the evolution of resistance (EPA 1998).

In high dose Bt corn, such as those that target European corn borer, the

mortality rate is nearly 100% (MarÇon et al. 2000), therefore survivors from the Bt crop

are extremely rare. The rootworm-targeted Bt hybrids currently on the market are all

low to moderate dose (EPA 2010 b,c, Hibbard et al. 2010b), so some WCR larvae are

expected to survive. The mortality rate of the Cry3Bb1-expressing corn hybrid

(Monsanto) is 98.6% therefore 1.4% of the rootworms are expected to survive (Hibbard

et al. 2010b). For Cry34/35Ab1 the mortality rate is 96.48% (Hibbard et al. 2010a), and

for mCry3A it is 94.88% (Hibbard et al. 2010b). The mortality rate of Smartstax® in the

environments reported to the EPA was 98.2% (EPA 2011a), which is actually less than

what was found for Cry3Bb1 by itself in a different set of environments. Clearly,

environmental conditions do play a role in the effectiveness of Bt relative to isoline

corn. Populations resistant to Cry3Bb1 (Meihls et al. 2008), Cry34/35Ab1 (Lefko et al.

2008), and mCry3A (Meihls 2011) corn have been established in laboratory selection

experiments within just a few generations. In the field, WCR resistance to Cry3Bb1 has

been documented across different parts of the Corn Belt including Iowa, Illinois,

Minnesota, Nebraska, and South Dakota (Gassmann et al. 2011,2012; Gassmann 2012,

Porter et al. 2012). The occurrence of WCR resistance to Cry3Bb1 has been attributed, in

part, to possible refuge compliance issues (Jaffe 2009, Gassmann et al. 2011) and the

repetitive use of the same management tactic (Gassmann et al. 2011, 2012; Gassmann

2012).

Cross resistance may occur when surviving one control measure allows the pest to survive another tactic at a higher rate than expected. Pyramided Bt products that include two or more Bt proteins that target a specific pest are becoming more widespread. One of the remedial actions being recommended after higher than expected damage has occurred in Cry3Bb1 fields is rotating to Smartstax®, incorporating Cry3Bb1+Cry34/35Ab1. Rootworm scientists warn that widespread use of pyramided corn hybrids, such as SmartStax®, in fields known to have resistance issues to Cry3Bb1, puts greater pressure on Cry34/35Ab1 (Porter et al. 2012). New pyramided and stacked Bt corn hybrids are in the pipeline for several companies, and having an understanding of cross resistance between Bt genes is vital for predicting resistance between these genes. Currently registered Bt pyramids all include the Cry34/35Ab1 gene including SmartStax, Agrisure 3122™ (Syngenta+Dow; mCry3A +Cry34/35Ab1), and Optimum AcreMax XTreme (Pioneer+Syngenta; Cry34/35Ab1+mCry3A). Agrisure Duracade™ incorporating eCry3.1Ab+mCry3A, which is Syngenta's next generation product, is expected to launch in 2014, pending final regulatory approval. Understanding cross resistance potential between Bt products using bioassays is a useful step in predicting cross resistance in the field between products. The objectives of this study are to assess cross resistance potential between mCry3A, Cry3Bb1 and Cry34/35Ab1 using the F1 generation of susceptible and field evolved resistant populations of WCR from Minnesota.

Materials and Methods

The experiment was conducted at the USDA-ARS facilities on the University of
Missouri campus in Columbia, MO. The experimental design was a 5 × 6 factorial with
five WCR populations and six maize lines (3 Bt and 3 isolines). Four of the WCR
populations were eggs taken from field collected wild adults from four locations in
Minnesota, and one population used was from a laboratory-raised diapausing colony
from Brookings, SD. The four locations of the wild-collected adults were from locations
near Dennison, Rosemount, Canby and Hills, Minnesota and are named with these
designations. The Dennison, Canby, and Hills, MN populations were all suspected to be
resistant to Cry3Bb1. The Rosemount, MN population was presumed to be susceptible
to Cry3Bb1, and the Brookings, SD diapausing population was field-collected prior to the
introduction of Bt corn and is a susceptible population. Ten replications were conducted
in a randomized complete block design. Larvae were recovered from seedling assays in
the growth chamber as well as from pots in the greenhouse and head capsule width and
dry weight were recorded along with larval numbers. In a separate set of pots in the
greenhouse, root damage ratings were recorded.

Rootworm Populations

The field history, planting dates and surrounding field characteristics are
important to consider at when trying to understand and predict the possibility of
resistance in the pest populations. All fields have a history of planting Cry3Bb1corn for
at least two years without rotation and have populations of northern and western corn

rootworms present. The Hills, MN field was on its second year of Cry3Bb1 corn when the adult beetles were collected. At this location, the grower practices a corn-corn-soybean rotation, but this field acts as a trap crop because the grower plants corn that has a relative maturity of 112-114 days compared to neighbors that have corn fields with a relative maturity of 102-103 days. This field has a block refuge configuration with beetles collected from the Cry3Bb1 corn. Adult rearing from this population yielded 26% NCR and 74% WCR. The Dennison, MN location is a long-term continuous corn field. In 2011, refuge was planted throughout the field as narrow strips from a ""split-planter" and had a block refuge previously. The rootworm beetles were collected within 300 feet of field border. The surrounding corn fields are primarily rotated in this area. Rearing the larvae from the Dennison, MN population yielded 62% WCR and 38% northern corn rootworms (NCR) beetles. The Rosemount, MN location is a long-term continuous corn field that has various planting dates. The rootworm adults were collected from refuge plants planted late. Adults reared from this population yielded 0.01% NCR and 99.99% WCR. About 1/3 of field is used for transgenic studies each year and the rest maintained as a refuge. The Canby, MN location is a field with at least seven years of corn on corn and has used a block refuge. The beetles were collected in the Cry3Bb1 portion of field. Corn following corn fields are more isolated in this region because most other fields are rotated with a non-corn crop. Rearing of larvae from the Canby, MN population yielded 16% NCR and 84% WCR beetles.

Eggs hatch rates were variable with Dennison, MN population, but this population was excluded from the seedling assays due to insufficient numbers of

healthy eggs. Additionally, the Rosemount, MN population was excluded from five reps

of the greenhouse assays due to a low numbers of eggs from this population at that

time. Approximate average egg hatch for all experiments were as follows: Rosemount:

61%, Canby, MN: 59%, Dennison, MN: 24%, Hills, MN: 59% and Brookings, SD: 76%.

The following are the coordinates and dates of collections:

- Hills MN - Collected Aug. 16, 2011. GPS Coordinates N43.53831 W096.39032
- Dennison MN - Collected Aug. 16-17, 2011. GPS Coordinates N44 21.033' W93 2.431'
- Madison MN - Collected Aug. 19, 2011. GPS Coordinates N44.8647 W96.1764
- Rosemount MN - Field D2/D3 Aug. 18-19, 2011. Not available
  Seedling assays

Each experimental unit consisted of a 15 × 10 cm plastic container (708 ml; The

Glad Products Company, Oakland, CA) holding 200 WCR eggs (~1-6-d-old) suspended in

a 0.15% agar solution. Containers with eggs were filled with 20 ml of water and ~150 ml

of a 2:1 mixture of autoclaved soil and ProMix potting soil (Premier Horticulture Inc.,

Quakertown, PA). After 1 wk, ~50 maize seeds (Bt or isoline) were added to containers

and covered with an additional ~300 ml of the soil mixture and 80 ml of water. All

containers were held in a controlled environmental chamber at constant 25°C and a

photoperiod of 14:10 (L:D) h. In addition, a subsample of eggs were dispensed onto

moist filter paper in a Petri dish at the same time as containers and placed in the

environmental chamber to estimate peak egg hatch. Three weeks following infestation

of eggs in containers and ~2 wk following peak egg hatch, larvae were recovered using

modified Berlese funnels equipped with a 60W incandescent light bulb. Recovery was

accomplished by cutting off the above ground plant tissue and emptying the container

contents into the funnel. Larvae were collected in half-pint mason jars filled with ~150 ml water that were attached to the bottom of the funnel. After 2 and 4 days, larvae were collected from jars and stored in 95% ethanol. Total number, dry weight, and head capsule width (HCW) were recorded for all recovered larvae.

Greenhouse assays

Corn plants were grown in the greenhouse at an average temperature of 25°C with a 14:10 (L:D) h schedule. Corn plants were grown in 3.8-liter plastic pots with stainless steel mesh (114-μm opening) screens (TWP, Berkeley, CA) hot glued over the drain holes to prevent larval escape. Two seeds were planted and then thinned to one upon germination. For all greenhouse assays, each pot was infested with 70 eggs when the plants were two weeks old. Peak hatch occurred approximately four weeks after planting time. Plants designated for evaluation of rootworm damage were grown until the V6 stage, which was approximately three weeks after peak hatch. After this, the roots were washed and then evaluated for root damage using the 0-3 node injury scale (Oleson et al. 2005). For larval recovery, approximately two weeks after peak hatch the corn plants were trimmed to the soil and the soil and roots were hung in mesh bags in the greenhouse where the temperature remained at ~30-35°C. As the soil dried, the larvae would migrate out and drop into a pan of water below as described in Hibbard et al. (2008). These larvae were collected twice a day for 10 days and stored in vials of ethanol for later processing. Total number, dry weight, and head capsule width were recorded for all recovered larvae.

Statistical Analysis

An ANOVA was used for the data analyses and was calculated by using the PROC MIXED of the SAS statistical package (SAS Institute 2008). For larval recovery, larval dry weight, head capsule width and plant damage, the linear statistical model contained the main plot effect of seed type, colony and their interaction. Although the figures show the untransformed data, data were transformed by square root (x+0.5) to meet the assumptions of the analysis. Replication was included as the random variable and all other variables were fixed. A separate analysis was done for plant damage rating, number of larvae recovered, larval head capsule and dry weights. Beyond the standard analysis of variance (ANOVA), we conducted preplanned comparisons of the treatment means between colonies within seed type and within seed type using the LSMEANS function in SAS.

Results

Seedling Assays

Overall, the four populations of WCR had variable larval recovery from all Bt hybrids, however the Canby and Hills, MN populations had higher recovery from Cry3Bb1 and mCry3.1Ab than the susceptible populations (Fig. 14). There was no difference between larval recovery of the Canby, MN population on mCry3A, Cry3Bb1 or Cry34/35Ab1 (Fig. 14). Across all populations, there was no difference in larval recovery from Cry34/35Ab1 for the seedling assays (Fig. 14). For the Canby, MN population, the

ratio of the larval survival on mCry3A and Cry34/35Ab1 hybrid to their isolines was larger in comparison to the ratio of Cry3Bb1 to Cry3Bb1 isoline (Fig. 14).

The HCW of the Canby and Hills, MN populations on Cry3Bb1 and mCry3A were significantly larger than HCW of the susceptible populations on those Bt hybrids (Fig. 15). The HCW of the larvae recovered from mCr3A were significantly smaller than the HCW of the larvae recovered from its isoline for each of the four populations (Fig. 15). There was no difference in the HCW between larvae from the Canby, MN population on Cry34/35Ab1 and its isoline, however the other populations did differ between these two corn types (Fig. 15).

Overall, the dry weight of the beetles recovered from all Bt hybrids were smaller than the beetles recovered from the isoline plants (Fig. 16). The highest average weights were from the Brookings, SD populations on isolines of Cry3Bb1 and Cry34/35Ab1 and Brookings, SD and Hills, MN on mCry3A isoline (Fig. 16). There were no significant differences in the dry weight of the larvae recovered from Cry3Bb1, Cry34/35Ab1 or mCry3A from any population (Fig. 16).

Greenhouse Assays

The Canby and Hills, MN beetles both had inflicted greater than a node of damage on Cry3Bb1 and mCry3A, but not on Cry34/35Ab1 (Fig. 17). For Cry3Bb1, the Canby and Hills, MN WCR populations caused significantly greater damage than the Brookings, SD and Rosemount, MN WCR populations (Fig.17). Although the Brookings, SD WCR population is presumed susceptible, it had a greater than 0.75 NIS damage

rating for mCry3A, which was not significantly different from the damage caused by the

Canby and Hills, MN WCR populations (Fig. 17). There was no significant difference

between the damage rating of Cry3Bb1 and its isoline when fed upon by the Canby and

Hills, MN WCR populations (Fig. 17). The damage ratings from the Canby and Hills, MN

WCR populations were not significantly different on Cry3Bb1 and mCry3A, however, the

damage to the mCry3A was significantly less than its isoline (Fig. 17). On mCry3A, the

Rosemount, MN WCR population caused significantly less damage than the other

populations on mCry3A (Fig. 17).

Overall, the larval recovery data from the greenhouse assays were more variable

than the seedling assay larval recovery data (Fig. 18). There was no significant difference

between any populations on Cry34/35Ab1 in terms of larval recovery (Fig. 18). The

Dennison, MN WCR population had significantly higher larval recovery on Cry3Bb1 than

the Rosemount and Brookings populations (Fig. 18). The Canby and Hills populations on

Cry3Bb1 plants had significantly more larvae recovered than the Brookings and

Rosemount populations (Fig. 18). There was no significant difference in the amount of

larvae recovered from mCry3A and its isoline for the Canby or Dennison, MN

populations (Fig. 18). Although the Rosemount, MN population is presumed susceptible,

there was no difference in larval recovery from mCry3A or its isoline plant, however the

Brookings, SD population had significantly less larvae recovered from mCry3A than for

the isoline in the greenhouse assay (Fig. 18).

There was no significant difference between populations within a corn type

(Table 10). The larval HCW of the Brookings and Rosemount populations were

significantly smaller on Cry34/35Ab1 compared to its isoline, however for these populations on mCry3A and Cry3Bb1 populations, there was no difference in HCW between each Bt and its isoline (Fig. 19). For the HCW of the Canby and Hills, MN populations, there were no significant differences between Cry3Bb1 and mCry3A and their paired isolines (Fig. 19). Overall, the dry weight of the larvae recovered from each Bt and its paired isoline was not significantly different, except for the larvae recovered from Rosemount and Hills populations on Cry34/35Ab1 (Fig. 20).

Discussion

Field populations from Canby and Hills that were presumed to have resistance to Cry3Bb1 had higher numbers of larvae recovered from both Cry3Bb1 and mCry3A plants than control populations, and root damage to the mCry3A plant by Canby and Hills populations was higher than expected. This damage was greater than 1 NIS and was not significantly different between these two populations on both mCry3A and Cry3Bb1. These patterns suggest cross resistance to mCry3A and Cry3Bb1 Bt hybrids exists for these populations. However, the ratio of survivorship on Cry3Bb1 to Cry3Bb1 isoline for the Canby population was smaller than on mCry3A to mCry3A isoline for the seedling assays, but not for the greenhouse assays. Also, damage to the mCry3A plant from the Canby and Hills populations were significantly less than the damage to mCry3A isoline plant, however this damage to the mCry3A plant from these populations was significantly greater than the damage from the susceptible Rosemount population on mCry3A. In both seedling and greenhouse assays there were equal larval recovery rates

across populations on Cry34/35Ab1, and there was equal damage (<0.5 NIS) to the Cry34/35Ab1 plants across all populations. Also, root damage was very low on Cry34/35Ab1 from the populations presumed resistant to Cry3Bb1, therefore, we found no apparent cross resistance between Cry3Bb1 and Cry34/35Ab1 or mCry3A and Cry34/35Ab1.

Even though no cross resistance has been observed between these proteins so far, the ability for rootworms to develop resistance to Cry34/35Ab1 is still a possibility if high selection pressure occurs. SmartStax as well as Agrisure 3122 have Cry34/35Ab1 proteins incorporated with a cry3 protein. If these pyramids are used in areas like Canby or Hills, MN, they will essentially be acting as a single hybrid because Cry3Bb1, and from what our data suggests, mCry3A may have little to no effect on them. This puts greater selection pressure on Cry34/35Ab1 in these situations especially if this method of control is used continuously (Porter et al. 2012). Possible resistance to mCry3A has been observed in Iowa recently, where Gassmann and Hodgson (2012) discovered several mCry3 and Cry3Bb1 fields that had higher than expected root injury (>1 NIS). Using an integrated approach to control the WCR is essential to slow the evolution of resistance to Bt hybrids like Cry3Bb1, mCry3a and Cry34/35Ab1. Soil insecticides are being used at a greater rate in conjunction with Bt, due to the Cry3Bb1 resistance problem (Gassmann et al. 2011). Prophylactic use of soil insecticides is occurring where rootworm infestations may not be high enough to warrant the application of insecticides (Porter et al. 2012). Insecticides are being applied on top of pyramided Bt hybrids with Cry34/35Ab1 proteins in areas where rootworm pressure may be high, however the Bt

proteins alone should be enough to reduce rootworm populations to acceptable levels. Gray (2011) suggests that rootworm thresholds are not being met by many farmers, and that they do not understand the rootworm pressure actually present. A major benefit of the adoption of Bt is the reduced use of insecticides, but with soil insecticides and foliar insecticides being used in conjunction with Bt, these benefits essentially disappear. The goal of IPM is to use management options in an integrated manner, not all at the same time as what some are calling the "kitchen sink" approach.

Overall, these data suggest the possibility of cross resistance between Cry3Bb1 and mCry3A, but not for all populations or in all assays. There could be inter-population variation in susceptibility to each protein from progeny of populations collected from the field. Some of the variation in this study may also be due to both NCR and WCR being present in different proportions in all populations. Populations as a whole were used and the proportion of NCR's that survived Cry3Bb1, mCry3A and Cry34/35Ab1 was not measured in this study. Future studies may rear the surviving rootworms to adulthood using on-plant assays and tease apart NCR and WCR survival from field collected populations. This study demonstrates the importance of using multiple assay types when comparing trends across data as results from assay types may vary due to plant age, Bt toxin concentration and available root material. Future pyramided hybrids should take into account cross resistance mCry3A and Cry3Bb1 proteins. Syngenta's new pyramid eCry3.1Ab will be stacked with a mCry3A protein, and we are currently assessing the possibility of cross resistance between these similar Cry3 proteins.

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Table 10. Significant factors of the seedling and greenhouse (G.H.) assays from five WCR populations and eight corn seed types.

	Effect	Num DF	Den DF	F Value	Pr>F
G.H. larval no.	colony	4	201	7.36	<.0001
	seed	5	201	8.01	<.0001
	seed*colony	20	201	2.16	0.0039
G.H. HCW	col	4	170	1.68	0.1566
	corn	5	170	6.6	<.0001
	col*corn	20	170	1.36	0.1494
G.H. dry wt.	colony	4	179	1.21	0.3072
	seed	5	179	4.14	0.0014
	seed*colony	20	179	0.91	0.5722
Damage	colony	4	260	9.2	<.0001
	seed	5	260	65.07	<.0001
	seed*colony	20	260	3.05	<.0001
Seedling larval no.	colony	3	161	2.46	0.0645
	seed	5	161	55.72	<.0001
	seed*colony	15	161	7.75	<.0001
Seedling HCW	col	3	157	6.16	0.0005
	corn	5	157	52.3	<.0001
	col*corn	15	157	5.31	<.0001
Seedling dry wt.	colony	3	153	4.62	0.004
	seed	5	153	44.22	<.0001
	seed*colony	15	153	2.28	0.0062

Figure 14. Number of larvae recovered from the seedling assays from each of the four colonies on eight corn types. The upper case letters indicate significant differences between corn types within population type, and lowercase letters indicate significant differences within corn type (*P*≤0.05).

Figure 15. Head capsule width (mm) (HCW) of larvae recovered from the seedling assays from each of the four colonies on eight corn types. The upper case letters indicate significant differences between corn types within population type, and lowercase letters indicate significant differences within corn type (*P*≤0.05).

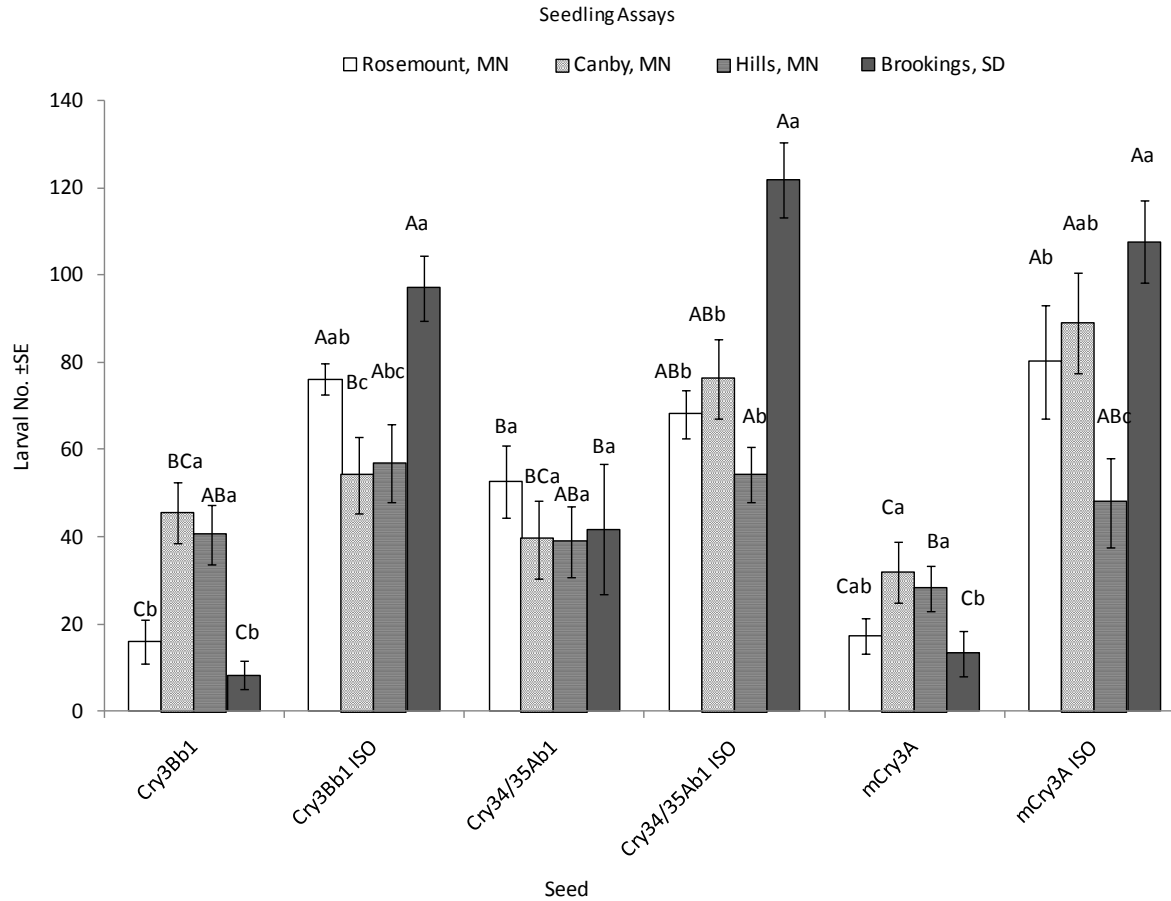Figure 16. Dry weight (g) of larvae recovered from the seedling assays from each of the four colonies on eight corn types. The upper case letters indicate significant differences between corn types within population type, and lowercase letters indicate significant differences within corn type (*P*≤0.05).

Figure 17. Root damage ratings of larvae recovered from the seedling assays from each of the four colonies on eight corn types. The upper case letters indicate significant differences between corn types within population type, and lowercase letters indicate significant differences within corn type (*P*≤0.05).

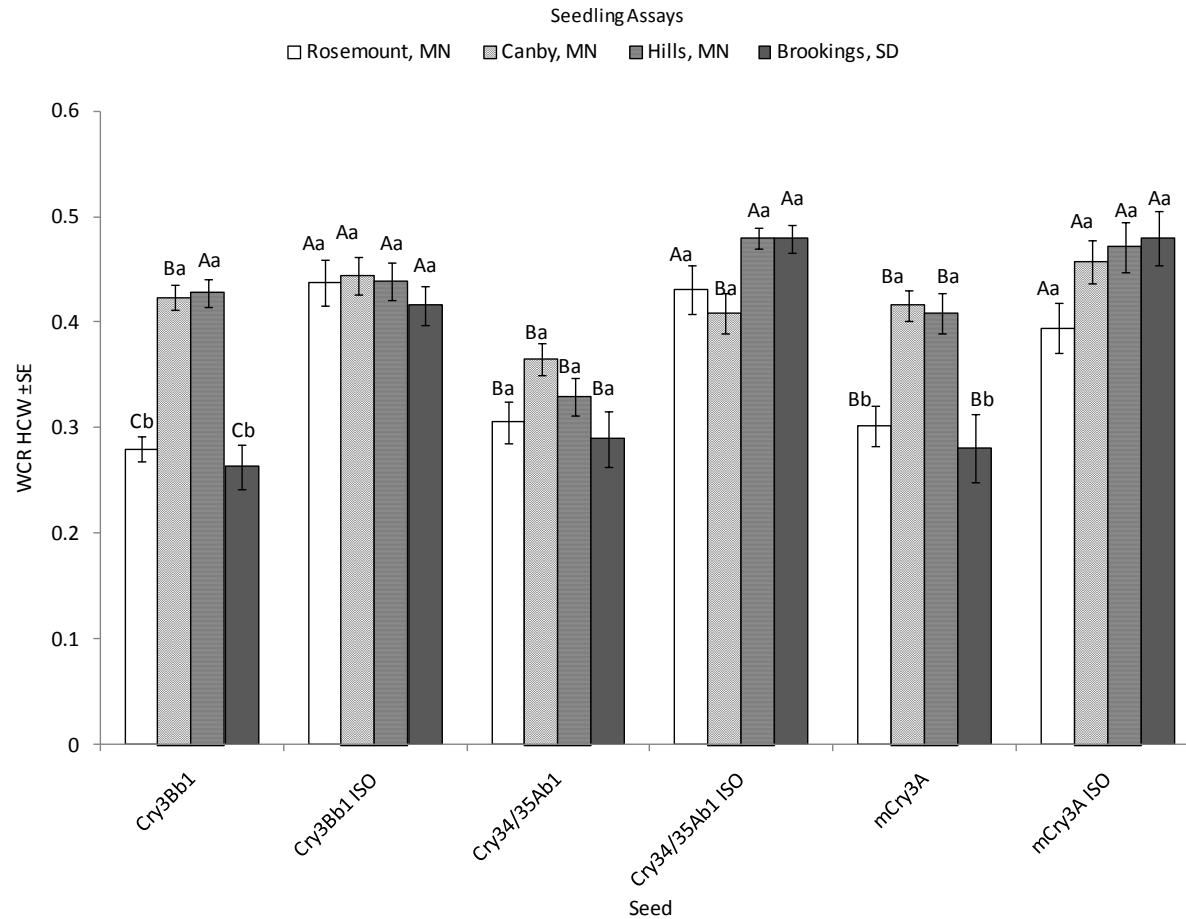Figure 18. Number of larvae recovered from the greenhouse assays from each of the four colonies on eight corn types. The upper case letters indicate significant differences between corn types within population type, and lowercase letters indicate significant differences within corn type (*P*≤0.05).
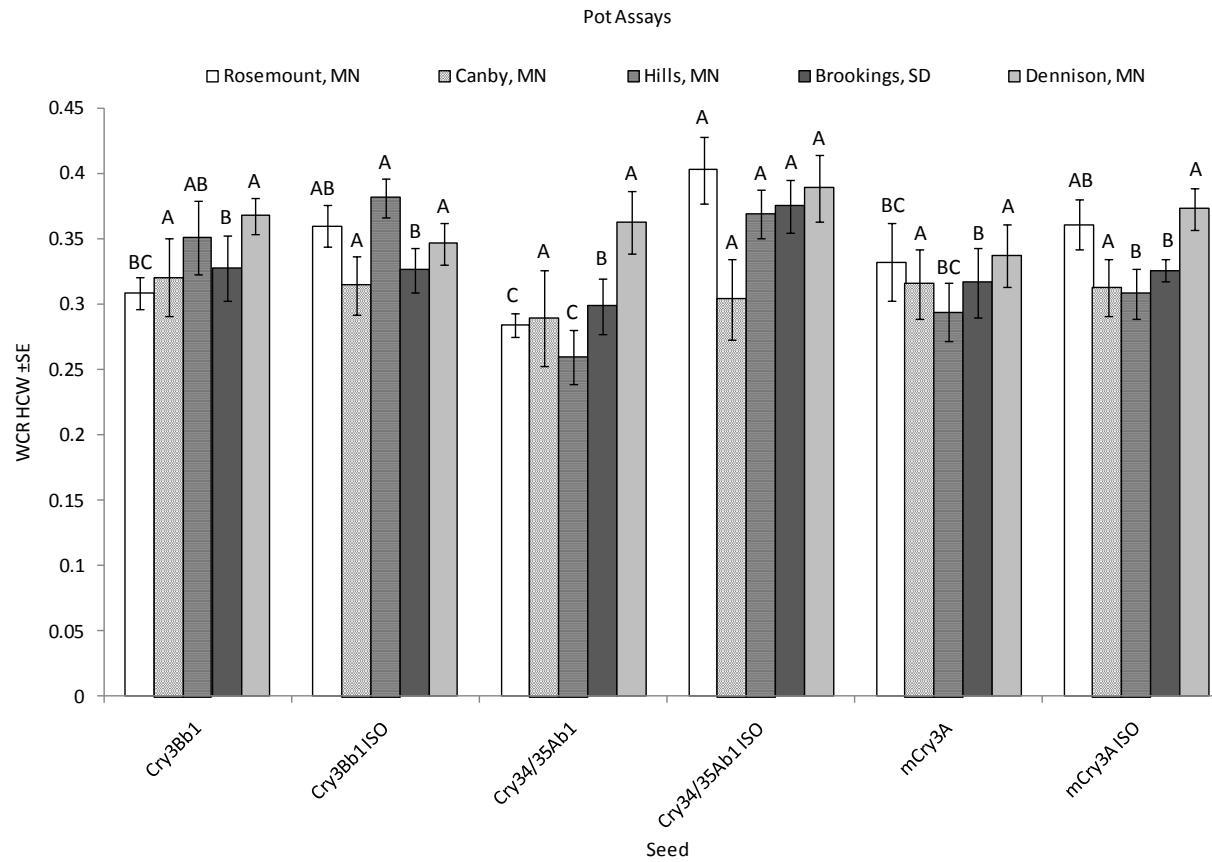
Figure 19. Head capsule width (mm) (HCW) of larvae recovered from the greenhouse assays from each of the four colonies on eight corn types. The upper case letters indicate significant differences between corn types within population type ($P \leq 0.05$).
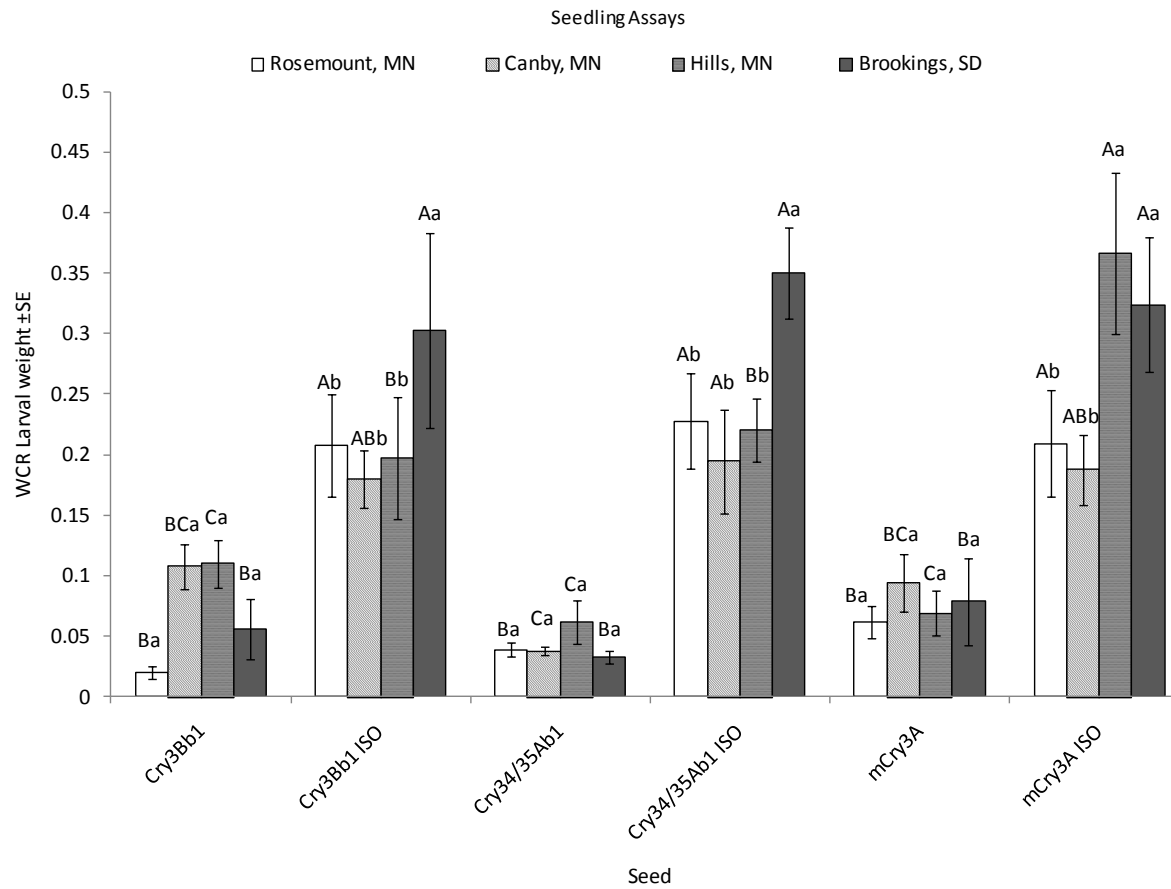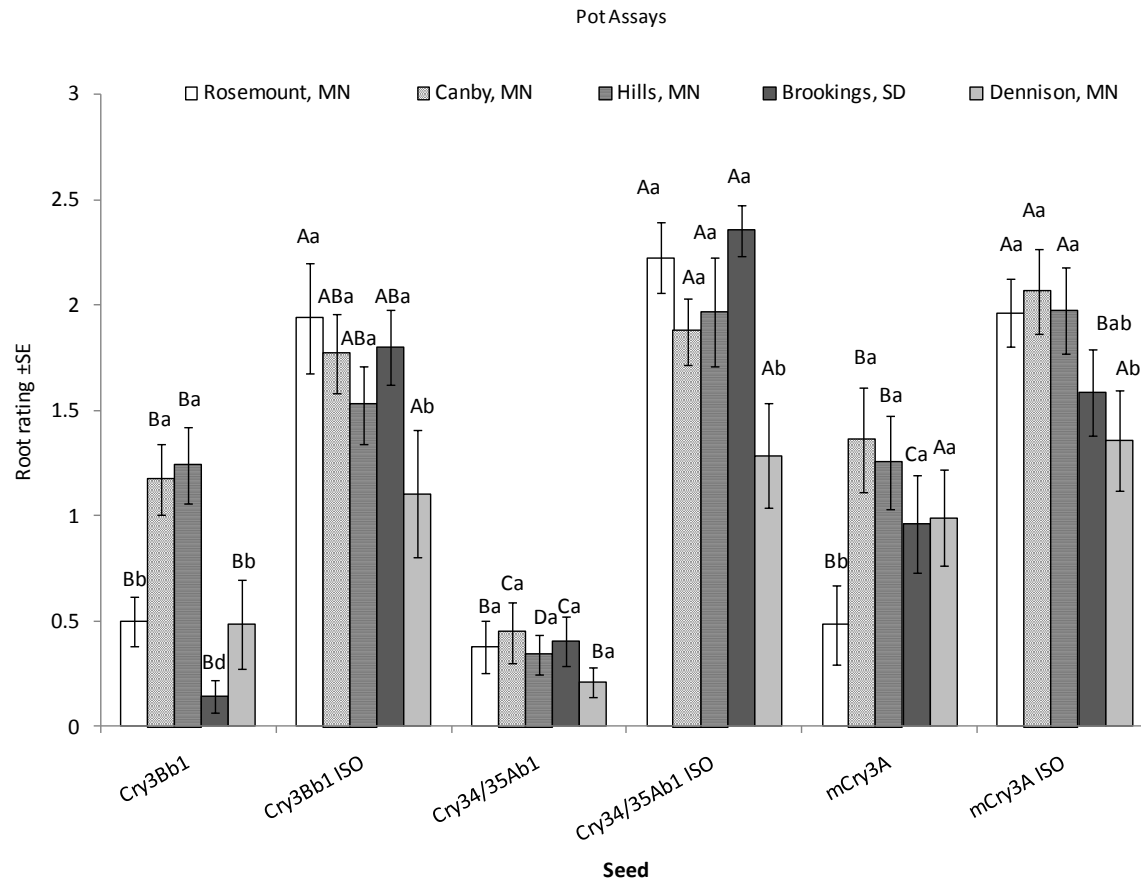
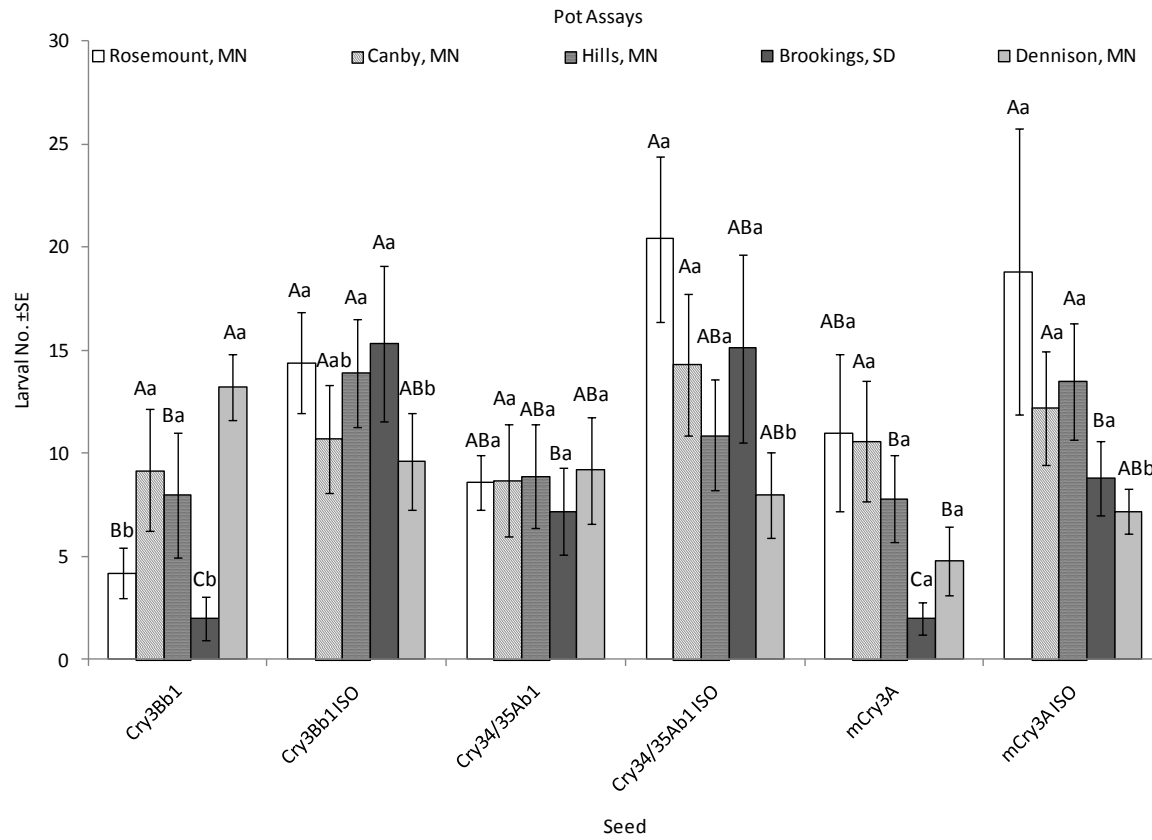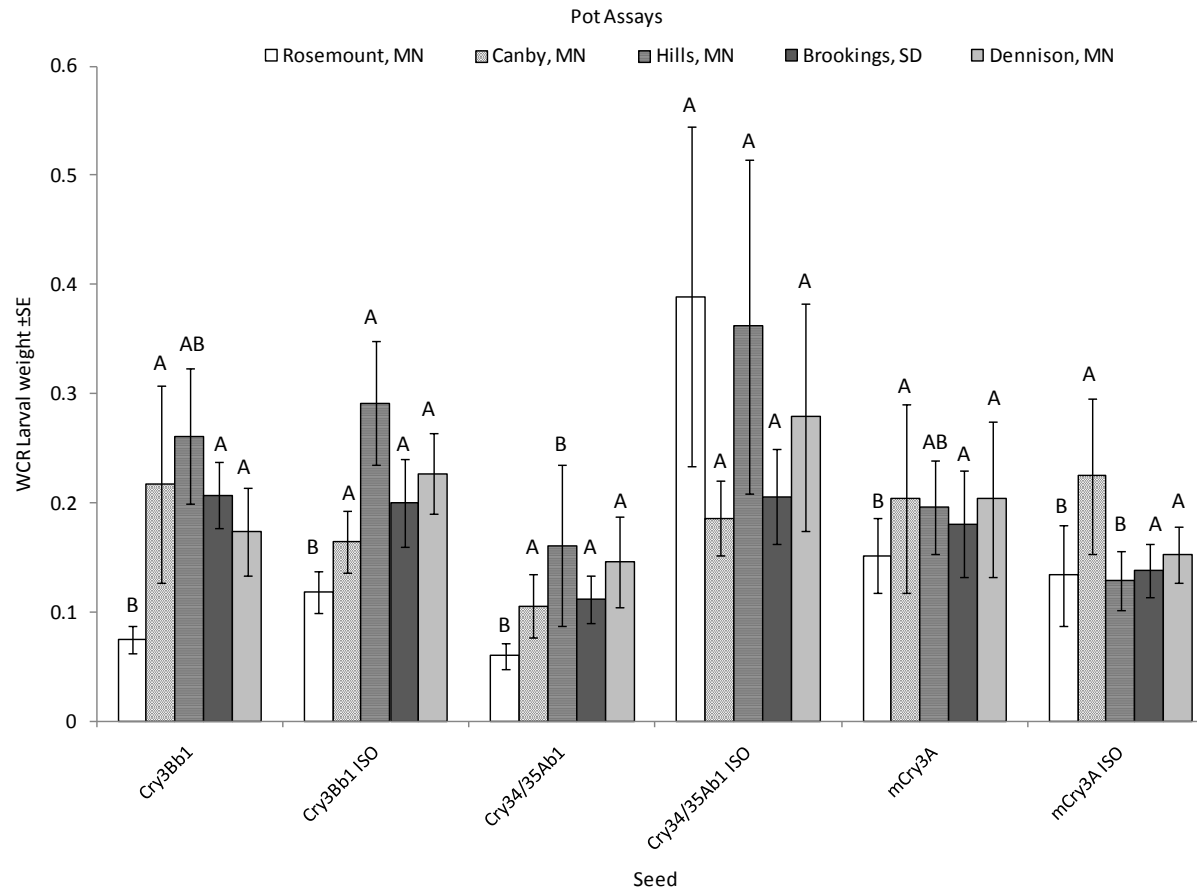Figure 20. Dry weight (g) of larvae recovered from the seedling assays from each of the four colonies on eight corn types. The upper case letters indicate significant differences between corn types within population type ($P\leq0.05$).

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VITAE

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