

Effect of nitrogen on Bt gene expression in corn roots, resulting trait performance against corn rootworms (*Diabrotica* spp.), and transgenic hybrid performance

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Dedication

This thesis is in memory of my father, Brad Franz. His inspiration allows me to give back to what he worked his whole life for: farming.

Abstract

This study examined the impact of nitrogen rates on the expression of corn rootworm (*Diabrotica* spp.) resistant traits in transgenic corn engineered to express one or more of Bt (*Bacillus thuringiensis* Berliner) Cry protein(s). The resulting protection against corn rootworm, and the comparative performance of hybrids containing zero, one or multiple (pyramided) corn rootworm traits were evaluated. Recommended nitrogen application rates have been suggested based on economics, but not for optimal expression of Bt Cry proteins. Specifically, this experiment explored the need to shift N rates to optimize Bt trait expression, corn rootworm protection provided by different traits, and a possible revision of nitrogen requirements for optimal yield with pyramided traits.

The experiment featured a factorial treatment arrangement in a split-plot randomized complete block design with six nitrogen rates as the main plots and three hybrids differing in corn rootworm traits as the sub-plots. Corn roots were sampled at the beginning of and just after peak larval feeding and run through an Enzyme-linked Immunosorbant Assay (ELISA) to determine gene expression levels. Resulting root injury and adult emergence were measured to assess impacts on larval survival and damage. Root injury generally decreased with nitrogen rate in all hybrids while Bt gene expression, for Cry3Bb1, increased. Nitrogen rate did not affect expression of Cry34 Ab1/Cry35Ab1. Below-ground biomass was found to be highest in the Round-UpTM Ready hybrid at growth stage V6, along with nitrogen uptake. Nitrogen uptake was similar in VT TripleTM to the Round-Up Ready hybrid, which were both significantly higher than the Smart StaxTM hybrid. Yield for the Smart Stax hybrid did not plateau with the nitrogen rates applied at one site in this study, while the unprotected Round-Up Ready hybrid could not take advantage of the higher N rates. Implications of these results

for growers in terms of adjusting nitrogen application rates in fields to obtain better gene expression, optimizing hybrid protection from corn rootworm traits, and reducing the risk of resistance are discussed.

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Chapter I.

Review of the Literature

a. Corn Rootworm (*Diabrotica spp.*)

Corn rootworm beetles (*Diabrotica spp.*), belonging to the family Chrysomelidae (Order: Coleoptera), have been pests of economic importance for over a century on *Zea mays* (corn). The two species commonly found in the northern Corn Belt are *Diabrotica virgifera virgifera* Leconte (Western Corn Rootworm) and *Diabrotica barberi* Smith and Lawrence (Northern Corn Rootworm). *Diabrotica virgifera virgifera* can be found throughout the Midwestern United States and Mexico (Krysan et al. 1980). *Diabrotica virgifera virgifera* is believed to have evolved with the domestication of corn and followed it into the United States, resulting in the beetle's high fidelity to corn. By contrast, *D. barberi*, which is native to the Corn Belt, is able to utilize alternate hosts more easily (Smith 1966, Branson and Krysan 1981). *Diabrotica virgifera virgifera* was first recognized as a U.S. pest in 1909 on sweet corn (Gillette 1912). Both species feed on corn roots during the larval stage which causes significant damage, via nutrient up-take interference and lodging, moisture stress, and secondary attack by pathogens (Kahler et al. 1985). The adults also feed on the plant's silks and pollen. When high numbers of adult beetles are present in a field, silk clipping can cause a reduction in yield due to reduced kernel number (Godfrey et al. 1993).

Corn rootworms are found throughout the United States in overlapping habitats. Two additional corn rootworms, not prevalent in the northern corn belt, are the Southern Corn Rootworm or Spotted Cucumber Beetle (*Diabrotica undecimpuncta howardi* Barber) and the Mexican Corn Rootworm (*Diabrotica virgifera zea* Krysan and Smith).

Identification

Diabrotica virgifera virgifera and *D. barberi*, while occupying the same ecological niche, are distinctly different in appearance. The adults of *D. virgifera virgifera* are yellow with black stripes running the length of the elytra and variations occurring between males and females. *Diabrotica barberi* appear pale when first emerged and then take on a green hue as feeding ensues (Krysan and Miller 1986). Males and females of both species can easily be distinguished by the presence of an extra sclerite plate present on the apex of the abdomen of the males (White 1977). The basitarsi pads are also a distinguishing feature (Hammack and French 2007). While females contain typical hairy tarsomeres, males exhibit a hairless oval patch on tarsomere 1, which is possibly used to secure copulation position on the female elytra. The eggs of the two rootworm species are also clearly different (Athey et al. 1964). Western corn rootworm eggs lack pits within the polygons while northern corn rootworm eggs contain 6-12 pits per polygon (Athey et al. 1964). Larvae are difficult to visually identify to species. Identification of these species during the larval phase is best done with molecular tools (Guss and Krysan 1972).

Life cycle

Corn rootworm beetles are univoltine insects that overwinter as eggs in the soil and hatch in the spring (Krysan 1982). Both species of rootworm undergo unique embryonic developmental phases, which may aid in allowing them to withstand cold temperatures. In both embryos, the germ band separates from the serosa and migrates into the yolk before going through diapause (Krysan 1972). Each species requires a period of dormancy (diapause) where they either experience an extreme chill, in the case of *D. barberi*, or a period time consisting of three to four months, for *D. virgifera virgifera* (Krysan 1978, 1982). The diapause period is followed by a quiescence period, which is influenced by moisture (Krysan 1978). Egg hatch rates significantly decrease at a temperature of -17.4°C for *D. barberi* and -12.0°C for *D. virgifera virgifera* or below as duration at that temperature increases and moisture levels decrease during winter months (Gustin and Wilde 1984, Ellsbury and Lee 2004). Ground cover and reduced tillage have

been found to increase egg survival in cold winters (Gray and Tollefson 1988a). Both *D. virgifera virgifera* and *D. barberi* eggs will hatch and larvae continue development at temperatures above 11.7°C (52°F) (Kuhlman et al. 1970, Wilde 1971).

Larvae respond to CO₂ signals and various chemical compounds released by corn roots, which guide them through the soil profile (Strnad et al. 1986, Bernklau et al. 2009). Carbon dioxide levels are such an important part of larvae orientation towards the root that in experiments where a more concentrated, synthetic, CO₂ source was placed away from corn roots, the larvae moved toward the non-biological CO₂ source instead (Bernklau et al. 2004). Larvae are able to detect small changes in CO₂ levels, which allows for more precision in seeking host localization (Strnad et al. 1986). When first instar larvae reach the roots of susceptible plants, they enter and migrate towards the tip due to higher concentrations of CO₂ and then burrow into the cortex of seminal roots (Strnad and Bergman 1987). Second and third instar larvae will redistribute within the root system and feed within older roots or on the outside of roots due to their size (Strnad and Bergman 1987). There is evidence of competition between *D. barberi* and *D. virgifera virgifera*. The larvae of *D. barberi* were displaced and had higher mortality rates when placed in a pot with *D. virgifera virgifera* (Piedrahita et al. 1985). High infestation rates result in limited food availability, which delays developmental times and increases density-dependent mortality during the larval stage (Branson and Sutter 1985, Weiss and Mayo 1985, Onstad et al. 2006). Root system injury ratings have concentrated on the 4th through 7th nodes due to the development of those nodes overlapping with egg hatch in rootworms (Strnad et al. 1987). Root pruning results in lower fresh and dry weight of roots (Gavloski et al. 1992). Larvae will remain in the soil and feed on roots for three to four weeks. Pupation occurs in soil cells where the beetle transformation takes approximately two weeks with no feeding or other external movements (Chiang 1973).

Larval densities also have an impact on adult size and head capsule width (Branson and Sutter 1985, Weiss and Mayo 1985). Higher densities stress larvae, which results in smaller adults, a higher ratio of males emerging, shorter lifespans and reduced fecundity.

Adult emergence differs slightly between sexes. Males emerge approximately five to seven days before females due to the need to complete development to sexual maturity (Branson 1987, Guss 1976) before mating. Average longevity is 56.6 days for females and 44.7 days for males (Ball 1957). Females emerge sexually mature and will mate as soon as they come into contact with a male. Males make more mating attempts, and have an initial preference for heavier females, while females only mate once (Kang and Krupke 2009). Males continue to mate with multiple females between July and August with copulation taking three to four hours each time (Ball 1957, Hill 1975, Lew and Ball 1979). Once mated, females continue to feed for a variable period of time before ovipositing eggs in the top 30 cm of moist soil or in cracks in the soil surface (Ball 1957, Kirk 1981). Mean lifetime fecundity of the female rootworms under optimal conditions was observed to be around 440 eggs (Boetel and Fuller 1997).

Adult movement, in the form of interfield flights, typically occurs during the early morning and evenings due to temperature (Isard et al. 2000). *Diabrotica virgifera virgifera* adults feed on corn silks and pollen primarily, but will also feed on corn leaves before and after pollen and silks are no longer available (Branson and Krysan 1981). *Diabrotica barberi* has similar food sources but is not known to feed on corn leaves (Branson and Krysan 1981). The limited food sources on corn, along with possible competition factors, causes *D. barberi* to leave corn fields once the pollen and silks have dried up (Branson and Krysan 1981). These feeding behaviors, coupled with *D. virgifera virgifera*'s competitive success in continuous corn fields, likely have a large influence on why *D. barberi* have higher populations in rotated fields and *D. virgifera virgifera* have larger populations in continuous corn fields (Hill and Mayo 1980).

Management

Crop rotation is a control tactic that has been effective for over 100 years. By alternating planting seasons with a non-host crop, rootworm eggs would hatch without a food source and die, resulting in population control (Levine and Oloumisadeghi 1991). *Diabrotica barberi* answered this control tactic by developing extended diapause in multiple states throughout the Midwest, which allowed them to remain dormant through the growing season in which the non-host was planted (Krysan et al. 1986). *Diabrotica virgifera virgifera* responded to crop rotation by laying eggs in soybean fields so the next growing season, when corn was planted, a suitable host would be available again (Levine and Gray 1996). The new egg-laying behavior is not due to attractants from other crops but instead is a result of a loss of fidelity to corn (Knolhoff et al 2006). Delaying planting date can be an effective form of cultural control as well, which reduces the amount of injury caused by the beetle due to inadequate food sources for the larvae after egg hatch (Bergman and Turpin 1984), but is an agronomic liability. Moldboard-plowing has also been used as a form of control due to the significant reduction in overwintering survival of corn rootworm eggs it causes while no-till and chisel-plowing do not effect survivability (Gray and Tollefson 1988).

Insecticides were a common form of control for many years and are still used today, targeting corn rootworms, but resistance has been formed in response to many of them, including foliar insecticides aimed at controlling adults (Wright et al. 2000, Meinke et al. 1998). The soil insecticides act to protect the corn root system from injury but were not always efficient at controlling corn rootworm itself (Levine and Oloumisadeghi 1991). Tolerance has been used as another form of management, but does not have an effect on the insect and instead refers to the plant's ability to generate a large root system and regrow roots after injury (Branson et al. 1982). The control tactics that have been implemented for the past 30 years in order to control the pest, have been overcome by the corn rootworm's (in some areas of the corn belt) adaptable life cycle (Meinke et al. 2009).

Chapter I. **Review of the Literature**

b. Corn Rootworm Resistant Transgenics

Historically, the corn rootworm has been one of the most damaging and economically important pests on corn (Gray et al. 2010). A high proportion of the corn grown in 2011 included corn rootworm resistant transgenic hybrids (USDA 2011). Corn rootworm resistant hybrids, producing *Bacillus thuringiensis* (Bt) Berliner, were first described by Moellenbeck et al. (2001) and in subsequent years by Ellis et al. (2002), and Vaughn et al. (2005). These transgenic corn hybrids were produced by using particle acceleration to insert the Bt genes into corn tissue for constitutive expression throughout the plant (Vaughn et al. 2005).

The first corn rootworm-active transgenic hybrids became commercially available in 2003 (containing event MON863), when they were released by Monsanto (Monsanto, St. Louis, MO), expressing the corn rootworm resistant toxin Cry3Bb1 (Vaughn et al. 2005). The event was modified and released by Monsanto in 2005, containing a modified version of the Cry3Bb1 toxin with an enhanced promoter (e35S) (known as event MON88017) (Nguyen and Jehle 2009, EPA 2010b). A dual-acting protein combination containing Cry34Ab1 and Cry35Ab1 (known as event 59122) was inserted into hybrids and was released in 2005 by Dow AgroSciences (Dow AgroSciences, Indianapolis, IN), which is also active on corn rootworms (Storer et al. 2006, EPA 2010a). In 2006, additional corn rootworm- active hybrids (containing even MIR604), producing mCry3A, were released by Syngenta (Syngenta Seeds Inc.) (EPA 2006). By 2009, the EPA approved hybrids pyramided with two corn rootworm resistant toxins Cry3Bb1 and Cry34/35Ab1 (EPA 2011). The pyramided hybrids were produced to reduced the risk of resistance forming in corn rootworm populations.

Bacillus thuringiensis, a soil bacterium, produces proteins that are toxic to larvae of lepidopterans, coleopterans, and dipterans. *Bacillus thuringiensis* subspecies *kumamotoensis*, which produces the toxic crystal protein during its sporulation phase, was first characterized and sequenced in 1992 by (Donovan et al. 1992) as a rootworm-active insecticidal strain. While the Bt toxin used for the control of corn rootworm is approximately eight times more effective than the wild type protein (Vaughn et al. 2005), the lethal concentration (LC₅₀) of Cry3Bb1 needed to cause mortality in 50% of a susceptible population ranges from 0.74 μg/ cm² to 9.20 μg/ cm² in field populations, which is far higher than lepidopteran-active Bt toxins (Siegfried et al. 2005). The range of lethal concentration is similar to the effective concentration (EC₅₀) needed to show developmental impacts on the larvae. Thus, Cry3Bb1 does not have significant sublethal effects on corn rootworm larvae (Siegfried et al. 2005). Larvae that survive on Cry3Bb1 have the same life expectancy and egg hatch rate as unexposed larvae (Mohammad et al. 2005). The large range in effective concentrations and lethal concentrations is due to the variance found in corn rootworm populations, which was observed in Illinois by Gray et al. (2007).

Expression

Bt protein expression occurs throughout the plant in all tissues throughout the growing season (Nguyen and Jehle 2009). Expression of these toxins is driven by the constitutive, root-enhanced promoter. Expression levels are highest in young plants and newly formed tissues and tend to decrease with age (Nguyen and Jehle 2009). Root tissue expression of modified Cry3Bb1 ranges from 129.7 – 40.3 μg/g dry weight between early vegetative growth and late reproductive stages, respectively (Ahmad et al. 2005, Nguyen and Jehle 2009).

The level of protein expression in plant tissue is best analyzed with a quantitative Enzyme-Linked Immunosorbant Assay (ELISA) (Grothaus et al. 2006). A double antibody sandwich ELISA uses a primary anti-body to capture the target antigen and a secondary antibody to detect the bound antigen (Grothaus et al. 2006). A standard curve,

which corresponds to standard of known concentrations, is used to quantify the amount of antigen present in a sample (Grothaus et al. 2006). Samples are soaked in buffer, homogenized and centrifuged. Supernatant from the samples is loaded into ELISA plates and quantified.

Mode of action

Larvae, when feeding on corn rootworm resistant transgenic hybrids, continuously sample root hairs and other areas of the root after initially ingesting tissue with the toxin but not actively feeding on the root tissue (Clark et al. 2006). Once larvae feed on the transgenic corn roots and ingest the Bt protein, the protein is deprotonated in the larval midgut. Effects of the toxin begin ca. 12 hours after ingestion (Kaiser-Alexnat, 2009, Moellenbeck et al. 2001). Midgut epithelial cells become affected after the protein binds to cadherin receptors, which are involved in cell adhesion (Sayed et al. 2007). Once the toxin binds and undergoes a conformational change, pores open up in the midgut, allowing material from the insect hemocel to leak into the midgut (Schnepf et al. 1998). The protease activity on the Cry protein in the midgut is correlated with pH (Kaiser-Alexnat 2009). Corn rootworms are susceptible to the toxin as larvae during the first, second and third instars, but are not susceptible as adults nor does it effect adult longevity, which is likely due to a difference in the pH or the insect gut proteases (Nowatzki et al. 2006). The toxin, also, does not affect larval growth at sub-lethal concentrations according to Siegfried et al. (2005), but this contradicts recent findings involving delays in emergence by Hibbard et al. (2011).

Insect Resistance Management

IRM plans have been developed since the implementation of transgenic varieties in order to minimize resistance from developing among natural populations (U.S. Environmental Protection Agency) (EPA 2005). Hybrids that do not express the corn rootworm transgenic traits (also known as non-Bt refuge corn) have been planted near corn rootworm transgenic corn since it was first commercialized in 2003. The purpose of this refuge corn is to encourage random mating between corn rootworm beetles that have developed on non-Bt hybrids and Bt hybrids, to minimize the risk of resistance in corn

rootworm. Larval movement to transgenic plants from infested refuge plants is not significant, allowing refuge to be effective (Hibbard et al. 2005). While no sub-lethal effects have been found, a delay in beetle emergence on corn rootworm transgenic hybrids has been observed and has become a greater area of focus recently since it may have implications for the assumption of random mating with refuge beetles. This delay has been observed in multiple studies ranging from eight days to two weeks (Murphy et al. 2010, Hibbard et al. 2011). A slight delay in emergence has been observed on Cry34/35 hybrids, with significantly more females emerging from the transgenic hybrids compared with their isoline (Storer et al. 2006). A delay in emergence from the transgenic hybrids may implications for Insect Resistance Management (IRM) plans.

In 2009, however, populations of corn rootworm were collected from fields in Iowa that showed characteristics of corn rootworm damage even though transgenic corn was planted. Gassmann et al. (2011) performed assays on these populations, which demonstrated that the insects have developed resistance to Cry3Bb1. Further investigation showed that three consecutive years of planting the Cry3Bb1 toxin contributed to resistance formation in natural populations in multiple areas of Iowa (Gassmann et al. 2011).

Corn

Corn is a monocotyledonous plant in the family Gramineae. Corn germinates and emerges between five days and two weeks after planting (Ritchie et al. 1997). After emergence, root initiation from nodes one through four will occur in the first four weeks, along with about four vegetative nodes, above ground. About this time, corn rootworm eggs hatch and larvae migrate to the roots via CO₂ and chemical signals from the corn (Strnad et al. 1986). Feeding on the corn roots then takes place on the 4th through 7th nodes of roots (Strnad et al. 1987). Roots in the seventh node are partially above the soil line, acting as brace roots.

Corn phenology has been shown to have a significant impact on western corn rootworm emergence (Hibbard et al. 2008). Adult emergence is higher when larvae develop on the corn during early growth stages (V4 to V11) than on late growth stage corn (VT to R1). When egg hatch occurs later in plant growth development (VT to R1), plant damage is lower than when egg hatch occurs earlier.

Chapter I.

Review of the Literature

c. Nitrogen Fertilization Effects on Corn and Corn Rootworm

Nitrogen rates have remained constant over the past 20 years while corn yields have increased. The nitrogen rate applied depends on several factors such as the previous crop and the nitrogen price/ crop value ratio (Kaiser et al. 2011). The maximum return to nitrogen (MRTN), which is the most profitable N rate based on the cost of N and the value of corn is commonly used when deciding the N rate for a particular field (Sawyer and Nafziger 2005). For the upper Midwest, MRTN rates have been found to range between 134 and 184 kg N per ha⁻¹ (136 and 176 lbs N/acre) (Sawyer and Nafziger 2005). Today, nitrogen is applied as anhydrous, urea, and as a blend with other nutrients in liquid form (Rehm et al. 2006).

Nitrogen rate affects corn rootworms as well. Beetle emergence increases with the application of nitrogen, which may be due to an increase in root proliferation (Spike and Tollefson 1988, Riedell et al. 1996). Pikul et al. (2005) demonstrated that at high nitrogen rates, more corn rootworms emerged from corn-soybean rotations than from corn-on-corn, but within corn-on-corn there was significantly higher emergence at elevated N rates as well. Root nitrogen content has been positively correlated with the insect's efficiency of conversion of ingested food, which may result in higher fitness of the insect (Moeser and Vidal 2004). Nitrogen and carbon content of plant tissue also can be considered a parameter for explaining herbivorous insect success (Moeser and Vidal 2004). Though beetle emergence has been shown to increase with high nitrogen, this increase does not correspond to higher lodging (a common result of injury from the

insect). Besides the root system size and architecture, the lack of correlation with lodging may be due to the plants increased ability to regrow roots due to greater nitrogen availability (Spike and Tollefson 1988). Spike & Tollefson (1988) also demonstrated that nitrogen fertilization and plant density have significant relationships to root injury and corn biomass production. High rootworm injury and nitrogen deficiency has also been shown to increase silking intervals, which can have an impact on grain yield in corn (Spike and Tollefson 1989). Grain yield is reduced under high infestation levels as shown by Riedell et al. (1996). Root regrowth, following root injury, has also been shown to have a negative impact on yield when moisture is adequate (Gray and Steffey 1998). Both agronomic and environmental factors influence corn's response to corn rootworm damage (Spike and Tollefson 1989).

Nitrogen deficiencies in corn can cause reductions in growth as well as reductions in protein synthesis. The level of nitrogen availability during early growth stages of the corn plant was shown by Bruns and Abel (2003) to have a direct effect on the amount of Bt protein production in the plant. Also, whole plant nitrogen levels are positively correlated with the amount of lepidopteran-active Bt protein (Cry1Ab). Lepidopteran-active Bt hybrids accumulate 11% more nitrogen than their isolines at growth stage V7, which is when heavy feeding by corn rootworm larvae occurs (Subedi and Ma 2007). Nitrogen uptake does not differ in plants that express the European Corn Borer- resistant Bt toxin versus their isoline hybrid (Ma and Subedi 2005). Plant nitrogen levels are greatest in fields that are not infested (no feeding) versus fields with significant feeding (Kahler et al. 1985). Alinia et al. (2000) found larval survival on non-Bt (rice) plants to be lower when no fertilizer was applied versus control plants with fertilizer.

Table 1: Cry3Bb1 expression in *zea mays* tissues during different growth stages.
 [Adapted from Nguyen & Jehle (2009)]

Cry3Bb1 expression levels (ug/g dry weight)			
Tissue	Growth Stage		
	BBCH 19 (V4)*	BBCH 30 (V6)	BBCH 63 (VT)
Root	129.7	99.0	65.8
Stalk	184.0	113.9	47.3
Lower Leaf		126.8	117.0
Upper Leaf		151.7	125.5

* Growth stages in parenthesis represent the equivalent Leaf Collar stage as described by Abendroth et al. (2011).

Chapter II.

Corn Rootworm Response to Single and Pyramided Event Hybrids under Different Nitrogen Rates

Introduction

Corn rootworms (*Diabrotica virgifera virgifera* Leconte and *Diabrotica barberi* Smith and Lawrence) have been pests of economic importance in the U.S. on corn (*Zea mays* L.) for over a century (Gillette 1912, Riley 1880). Injury to the plant occurs when larvae feed on and within the roots of the corn (Strnad & Bergman 1987). Damage from corn rootworm feeding occurs in the form of nutrient up-take interference, lodging, secondary attack by pathogens, and drought stress (Kahler et al. 1985). To prevent this damage, several control tactics (e.g. including foliar/ soil insecticides and crop rotation) have been developed for corn rootworm (Wright 2000, Levine 1991). The most recent control tactic brought to market is transgenic corn producing toxins from the bacterium, *Bacillus thuringiensis* Berliner (Bt), for the control of corn rootworms (Bt corn). These toxins, expressed in the roots of Bt hybrids, results in mortality of the rootworm larvae after ingestion (Donovan et al 1992).

Currently available Bt hybrids contain either single resistance traits (one gene coding for Bt toxin production), or pyramided resistance traits. The first transgenic Bt hybrids (containing event MON863), producing Cry3Bb1, with activity on corn rootworms, were commercially released in 2003 (EPA 2003, Vaughn et al. 2005). The Bt hybrids sold today, marketed with event MON88017, produce a modified version of Cry3Bb1 (event MON863) for the control of corn rootworm (Nguyen and Jehle 2009, EPA 2010b). A dual-acting protein combination containing Cry34Ab1 and Cry35Ab1, marketed as event DAS-59122, was approved for release in 2005, which is also active on corn rootworms (Storer et al. 2006, EPA 2010a). The dual-acting protein (Cry34/35) subsequently was pyramided with Cry3Bb1 to create pyramided hybrids in 2010 (EPA 2011), which offer more forms of the toxin to the corn rootworms, resulting in higher efficacy and prolonged product life.

Bt genes, which are constitutively expressed help reduce injury and stress to the corn plants caused by the corn rootworms, but additional nitrogen may be required for optimum performance of these Bt traits. Optimal nitrogen rates are currently selected based on economic factors with no consideration of how these rates affect transgenic performance against corn insects. Current recommended rates for productive corn-on-corn fields in Minnesota range between 134-184.8 kg/ha (Kaiser et al. 2011). A potential, negative aspect of high nitrogen rates is increases in beetle emergence have been correlated with the application of nitrogen (Spike and Tollefson 1988), which may be due to an increase in root proliferation (Riedell et al. 1996). Root nitrogen content has also been positively correlated with the insect's conversion efficiency of ingested food, which may result in higher fitness of the insect (Moeser and Vidal 2004). While nitrogen fertilization appears to benefit corn rootworms, the effects on performance of Bt hybrids, from yield and trait efficacy perspectives, are unknown. This study examines the impact of nitrogen rates on the expression of corn rootworm resistant (Bt) traits in transgenic corn, the resulting root protection, and its effects on corn rootworm populations. Specifically, we hoped to learn if nitrogen rates need to shift to optimize Bt trait expression and the resulting corn rootworm protection provided by different traits.

Materials and Methods

Plot Design

Nitrogen and Bt corn effects on corn production and corn rootworm efficacy were studied in two fields at Rosemount, MN in 2011. The soil at this location is a Waukegan silt loam soil that is highly responsive to nitrogen fertilization and underlain by a sand-gravel layer that can accentuate drought stress. Fields for this study were selected based on two criteria: continuous corn production history without long-term use of transgenic Bt corn and heavy, natural rootworm pressure. The experiment featured a factorial treatment combination of nitrogen rates and Bt traits arranged in a split-plot design. Six nitrogen rates (0, 55, 112, 168, 224, 280 kg N per ha⁻¹) comprised the whole plots with three corn hybrids (DK44-60 - Round-Up™ Ready, DK44-90 -VT™ Triple Pro, and DK44-92 -

Smart Stax™) (Monsanto, St. Louis, MO; Dow AgroSciences Indianapolis, IN) as subplots. These hybrids differ in their corn rootworm expression by producing the following respective trait(s): none, Cry3Bb1, and Cry3Bb1 + Cry34/35Ab1. Each treatment combination was replicated 4 times with each subplot containing eight rows (76.2 cm row-spacing) seeded at a rate of 85,185 seeds per hectare. A 3.05 m buffer zone was added between each main plot in a bed to allow nitrogen rate transitions to be completed between main plots during granular urea application with a small plot fertilizer applicator.

Nitrogen Application

Nitrogen was broadcast in the form of Urea (46-0-0 N-P-K). Rates were chosen based on current recommendations from the University of Minnesota Extension (Kaiser et al. 2011). Additional rates above and below those recommended were chosen in order to get a full response curve. Sulfur (granular form) was added at a rate of 28 kg per ha⁻¹ to each plot as recommended (Kaiser et al. 2011). Non-studied nutrients (P, K, and S) were applied at non-limiting rates. Study sites were disc chiseled prior to fertilizer application and tilled after applications to incorporate fertilizer before planting.

Whole-plant Sampling

Six plants at growth stages V6 and V12 were randomly chosen from each plot (three each from rows three and six, respectively) based on a representative appearance in the interior of each plot (least 61 cm from the end of the row). Plants were removed with a round-point shovel inserted at least 20 cm from the base of the corn plant in order to gather as much fine root tissue as possible. The plants were then immediately washed off using pressurized water spray to remove soil, mucilage coating the roots and other organic debris.

Biomass Analysis

Plants were measured for above-ground height (base of plant to extended leaf tip) and then separated into above and below ground portions by cutting with a sterile razor blade approximately 2.54 cm from the base of the plant. The above-ground biomass samples, comprising the bulked tissue of six plants per plot, were placed in paper bags for drying and processing. Biomass samples were left in a drier at 65°C for 24 – 48 hours and the dried composite samples were weighed. Above-ground tissue samples were then ground using a Willey mill (Thomas Scientific) and passed through a 2mm sieve. Total nitrogen content was measured using a Variomax CN Combustion Analyzer (Elementars America).

Beetle Mortality measures

Adult beetle emergence was used as an indicator of trait efficacy in each sub plot. Six cut-plant emergence cages (Chaddha et al. 1993) were constructed and placed in each subplot (three per row in rows two and seven respectively). These cages cover an area that is 30.5cm (adjusted to reflect seeding rate) by 76.2cm. Cage locations were chosen based on plant spacing (~ 15cm apart) to ensure reproducible absolute sampling. Beetles were collected from cages every three to four days over the entire emergence period.

Root Injury evaluations

Corn roots were dug in mid-August to evaluate root injury from corn rootworms. Shovels were inserted a distance of approx. 20.32 cm away from the plant base and were driven straight down to reduce man-made injury to the root system. Removed roots were washed with a pressurized water system to remove as much soil and root-coating mucilage as possible. Roots, pruned to 3.81cm or less, were counted for each plant's root system as a measure of corn rootworm injury. These counts, rather than the Nodal Injury Ratings (Oleson et al. 2005), were analyzed directly because the converted NIR ratings did not meet assumptions for statistical analyses even when transformed.

Data analyses

Data were analyzed with an ANOVA in R (Team, 2011). Beetle data from both sites 1 and 2 were transformed on a natural log scale to meet necessary statistical assumptions. Regression analyses were used with the beetle data to find the variables that best explained the response seen with beetle emergence. Nitrogen, nitrogen uptake and percent nitrogen were checked due to their influence on beetle survival and fitness (Moser & Vidal 2004). Hybrid effects and below-ground biomass were checked due to their direct influence on beetle mortality and food availability. Percent nitrogen and below-ground biomass data were gathered at growth stages V6 and V12, but only V6 data were used for describing beetle emergence due to the majority of larval feeding occurring at this stage.

Root injury data were transformed to meet the assumptions for ANOVA with site 1 requiring a natural log transformation and site 2 requiring a square root transformation. Mean comparisons were performed using Fisher's LSD.

Results

Beetle Response

Beetle emergence differed among hybrids for site 1 ($F=127.36$; $df=2, 36$; $p<0.0001$) and site 2 ($F=98.57$; $df=2, 35$; $p<0.0001$). The unprotected Round-Up Ready (RR) hybrid produced the highest beetle emergence, followed by the near-isogenic VT Triple (VT3), and Smart Stax (SSX) hybrids. Efficacy (% reduction in beetle emergence from RR levels) for VT3 and SSX hybrids are reflected in Table 2. Apparent field efficacy averaged 78% at site 1 and 66% at site 2 for the VT3 hybrid and 91% at site 1 and 84% at site 2 for the Smart Stax hybrid. Beetle emergence did not increase with nitrogen rate (Table 2) at either site 1 ($F=2.18$; $df=5, 15$; $p=0.111$) or site 2 ($F=1.43$; $df=5, 15$; $p=0.268$).

Three variables had a negative effect on beetle emergence at site 1: nitrogen (kg^{-1}) within above-ground biomass, hybrid VT Triple Pro and hybrid SSX (Table 3). Site 2 had a similar response in adult emergence to the hybrids but not with percent nitrogen. Below-ground biomass at growth stage V6 and RR had a positive effect with beetle emergence in both sites 1 and 2 (Table 3). At site 2, nitrogen uptake also had a positive effect on beetle emergence.

Root Injury

Root injury significantly differed among nitrogen rates ($F=11.29$; $df=5, 15$; $p=0.0011$) and hybrids ($F=749.82$; $df=2, 33$; $p<0.0001$) for site 1 (Fig. 1). A decrease in root injury was observed between the RR hybrid and the Bt hybrids and with increasing nitrogen rates. Consequently these data are presented in separate graphs (Fig. 1), differing in y-axis scales in order to show the relationships among Bt hybrids that would otherwise be masked by the higher injury levels observed in the control treatment. Bt traits achieved a 96% reduction in root injury at site 1 with the VT3 hybrid and 98% with SSX.

Root injury significantly differed among hybrids ($F=393.69$; $df=2, 34$; $p<0.0001$) for site 2 but not nitrogen rates ($F=2.03$; $df=5, 15$; $p=0.1319$) (Fig. 2). The RR hybrid had the highest number of roots pruned to 3.81cm or less, which was significantly different from hybrids, VT3 and SSX. There was a 99% reduction in root injury for both the VT3 and SSX hybrids at site 2.

Discussion

The results gathered from this study suggest nitrogen rates of 168 - 224kg/ha optimize beetle control and minimize root injury in corn plants at site 1, while site 2 had inconsistent results, not allowing a recommendation to be made. Beetle emergence differed significantly among the two transgenic hybrid treatments in this study, with considerably fewer occurring in the SSX hybrid than VT3 Triple hybrid, indicating a performance difference between these hybrids. In site 1, efficacy improved 13% with a

pyramid of two Bt traits compared to 18% in site 2 (Table 2). The increase in efficacy suggests that combination of Bt events in one hybrid is more effective than one event in a hybrid. This observation is consistent with other Bt studies performed by Ostlie et al. (2010- unpublished data) in MN.

The lack of significance in nitrogen rate effects on beetle emergence was unexpected considering an increase in percent control with increasing nitrogen rates for the hybrid VT3 (Table 2). For site 1, percent nitrogen found in the plant at growth stage V6, had a negative effect on beetle emergence. The finding that beetle emergence declines in the transgenic hybrids as percent nitrogen in the plant increases contradicts the study by Moeser and Vidal (2004), which found that increases in plant nitrogen content help an insect's efficiency of conversion of ingested food in non-Bt plants. A presumed explanation for this difference is that increases in Bt protein production as plant nitrogen levels increase result in reduced beetle emergence. Both sites 1 and 2 demonstrated a positive relationship between beetle emergence and higher below-ground biomass at growth stage V6, which can be explained by greater colonization of the root system and higher availability of food for the larvae, especially in the Round-Up Ready hybrid. Higher amounts of below-ground biomass tissue may also reduce density-dependent mortality in the beetle population (Hibbard et al. 2010).

Root injury was found to decrease with increasing nitrogen rates for site 1 in this study. There are a number of possible explanations for this finding, including: better beetle control due to an increase in Bt production, larger root-systems that dilute colonization or increasing root re-growth with higher nitrogen availability. Regrowth has been shown to increase with higher amounts of available nitrogen (Spike and Tollefson 1988). In site 2 there was not a steady response to nitrogen rate. Differences in responses between site 1 and site 2 are likely due to the temporary flooding during a heavy rainfall at site 2 during growth stage V4 (Fig. 3), which may have resulted in higher than normal mortality in the corn rootworm population at that site. Nitrogen leaching at growth stage V4 could have also resulted in less root re-growth. Macdonald and Ellis (1990) found larval movement

to be highly restricted in wet soil or very dry soil, which may have been a factor in this study as well. Whatever the mechanism, root injury due to beetle emergence was not consistent in this study. A trend in increasing beetle emergence with increasing nitrogen rate was found in the RR hybrid, while nitrogen rate had an opposite effect on root injury. The increase in beetle emergence with a decrease in root injury may be explained by the quality of food or an increase in root biomass. Nitrogen rate increased the percent nitrogen in corn roots, resulting in a higher quality of food for the beetles, requiring less feeding (Moeser and Vidal 2004).

Nitrogen rates did not have a significant influence on corn rootworm emergence in this study; further studies are needed to evaluate nitrogen rates and the effect they have on transgenic hybrid performance under different conditions. The lack of effect on beetle emergence due to nitrogen rate is a positive outcome due to the high demand for this input in the corn system. With a significant decrease in beetle emergence occurring from VT Triple Pro to SSX, that pyramided hybrid options for corn rootworm control should be considered before the use of this single-trait hybrid. The response to nitrogen rates and hybrid may vary under different soil conditions prone to flooding, leaching, or drought and as many of these environments as possible should be explored in order to fully understand how these transgenic hybrids perform under variable conditions.

Table 2. Efficacy of Bt hybrids (VT™ Triple, Smart Stax™), as measured by percent reduction in corn rootworm emergence from unprotected, isogenic Round-Up™ Ready hybrid under different nitrogen rates. Studies were conducted at the Rosemount Agricultural Experiment Station in 2011.

Site 1			
N Rate	Beetles from RR* (1000's/ha)	% Reduction** RR: VT3	% Reduction RR: SSX
0	251.63	75	92
55	217.11	72	88
112	380.36	78	91
168	430.52	80	90
224	370.01	83	90
280	337.61	79	94
Avg.	331.21	78	91
Site 2			
0	153.19	51	74
55	197.59	58	82
112	275.45	72	88
168	280.68	72	89
224	300.79	72	86
280	280.15	71	84
Avg.	247.98	66	84

* Round-Up™ Ready hybrid (control).

** % reduction from unprotected RR hybrid.

Table 3. Regression terms explaining variability found in corn rootworm beetle emergence from the nitrogen-Bt hybrid studies conducted near Rosemount, MN in 2011.

Site 1					
	<u>Intercept</u> (1000s/ ha)	<u>SE</u>	<u>t-value*</u>	<u>Pr(> t)</u>	<u>Adj. R²</u>
<u>Beetles</u>					
Round-Up Ready hybrid	3.772	0.102	37.026	<0.0001	0.78
VT Triple Pro hybrid	-1.416	0.144	-9.825	<0.0001	0.78
Smart Stax hybrid	-2.282	0.144	-15.836	<0.0001	0.78
V6 Below-Ground Biomass	1.211	0.233	5.207	<0.0001	0.28
%N in biomass ** @ V6	-0.399	0.171	-2.326	0.023	0.06

Site 2					
	<u>Intercept</u> (1000s/ha)	<u>SE</u>	<u>t-value*</u>	<u>Pr(> t)</u>	<u>Adj. R²</u>
<u>Beetles</u>					
Round-Up Ready hybrid	3.488	0.083	42.055	<0.0001	0.76
VT Triple Pro hybrid	-1.034	0.117	-8.815	<0.0001	0.76
Smart Stax hybrid	-1.755	0.119	-14.802	<0.0001	0.76
V6 Below-Ground Biomass	0.985	0.118	8.330	<0.0001	0.50
V6 N Uptake	0.002	0.001	2.219	0.0299	0.06

* Assuming null hypothesis that the estimate is zero; no relationship.

** % N in above-ground biomass

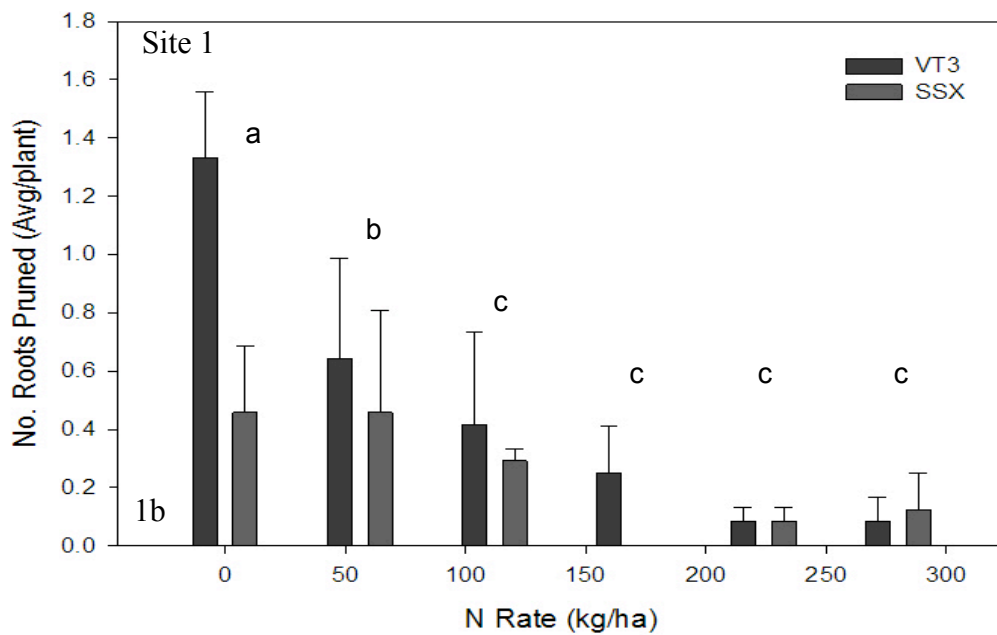
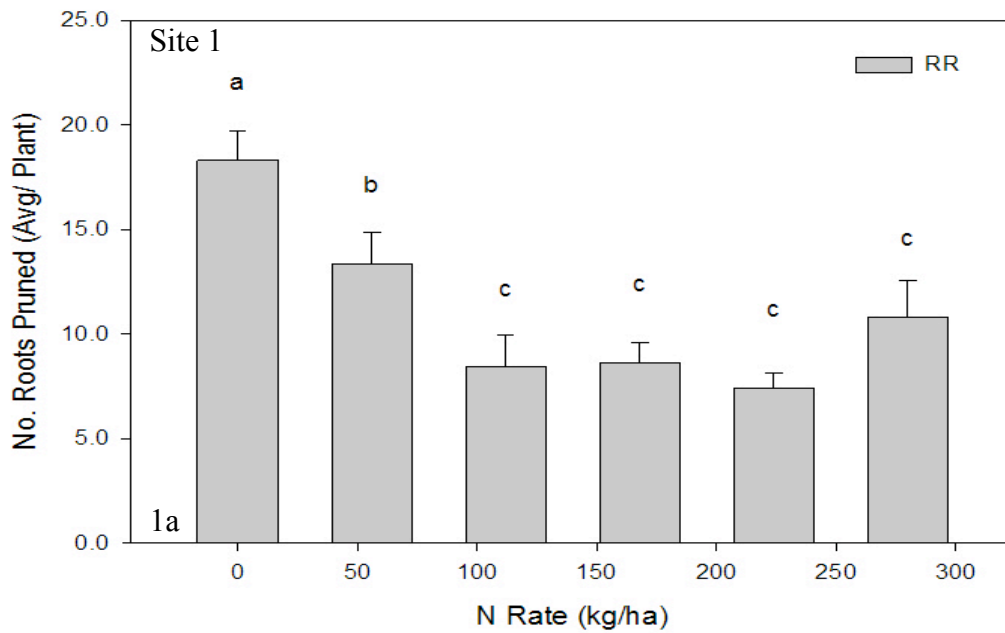


Figure 1. Corn rootworm injury (mean number of roots pruned to ≤ 3.81 cm) for hybrids differing in Bt protection as it relates to nitrogen rate applied (N: $p=0.0001$; Hybrid: $p<0.0001$) (LSD for N = 0.188). Fig. 1a. RR (control) hybrid. Fig. 1b. VT Triple (VT3) and Smart Stax (SSX) hybrids. Y-axis adjusted to reflect counts of injured roots between graphs 2a and 2b. Letters represent mean separation results for N Rate. Site 1. Rosemount, MN 2011.

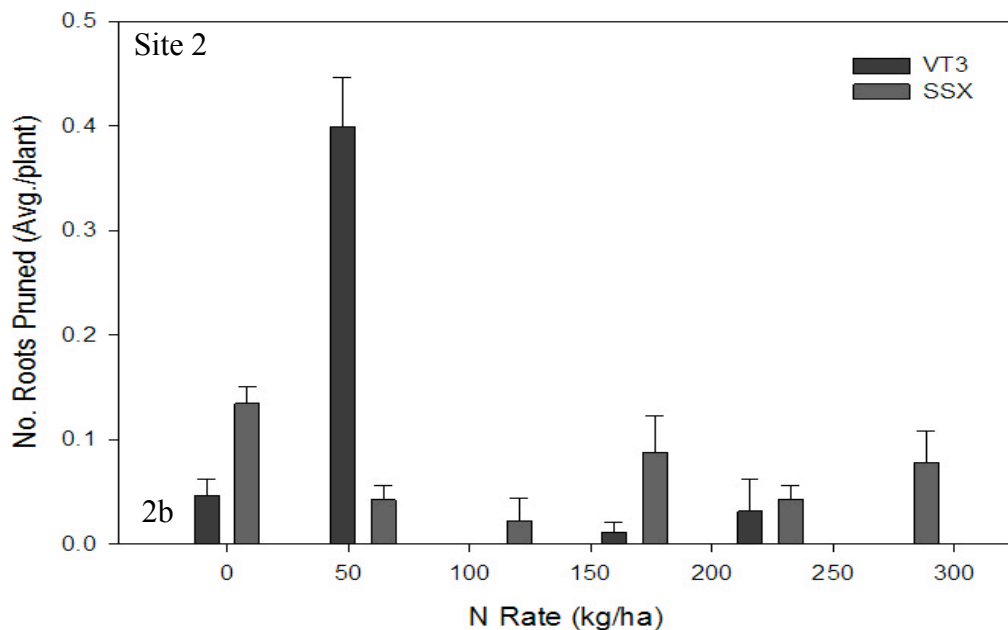
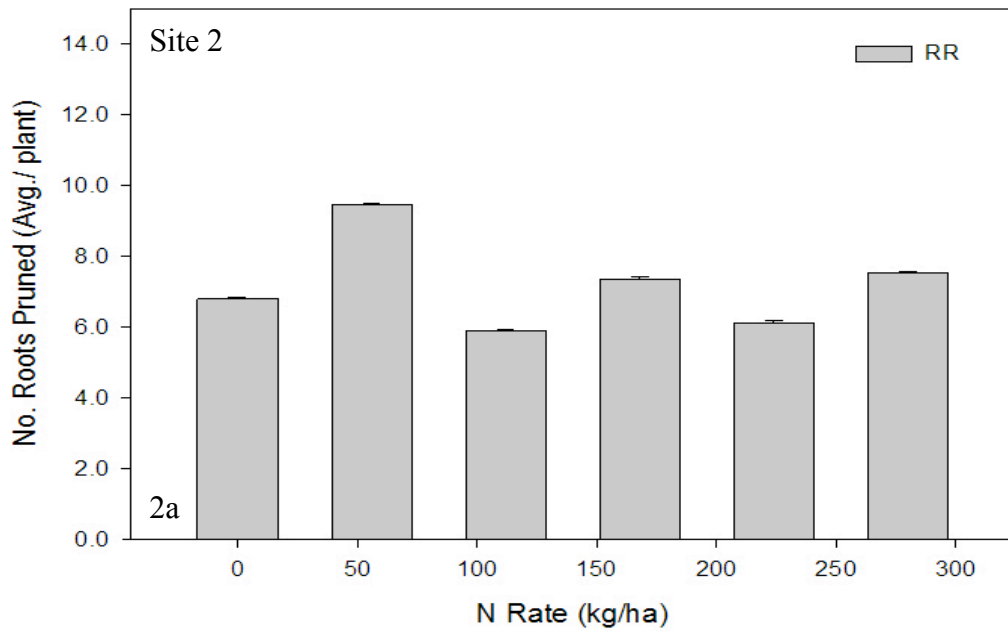


Figure 2. Corn rootworm injury (mean number of roots pruned to ≤ 3.81 cm) for hybrids differing in Bt protection as it relates to nitrogen rate applied (N: $p=0.132$; Hybrid: $p<0.0001$). Fig. 2a. RR (control) hybrid. Fig. 2b. VT Triple (VT3) and Smart Stax (SSX) hybrids. Y-axis adjusted to reflect counts of injured roots between graphs 2a and 2b. Site 2. Rosemount, MN 2011.

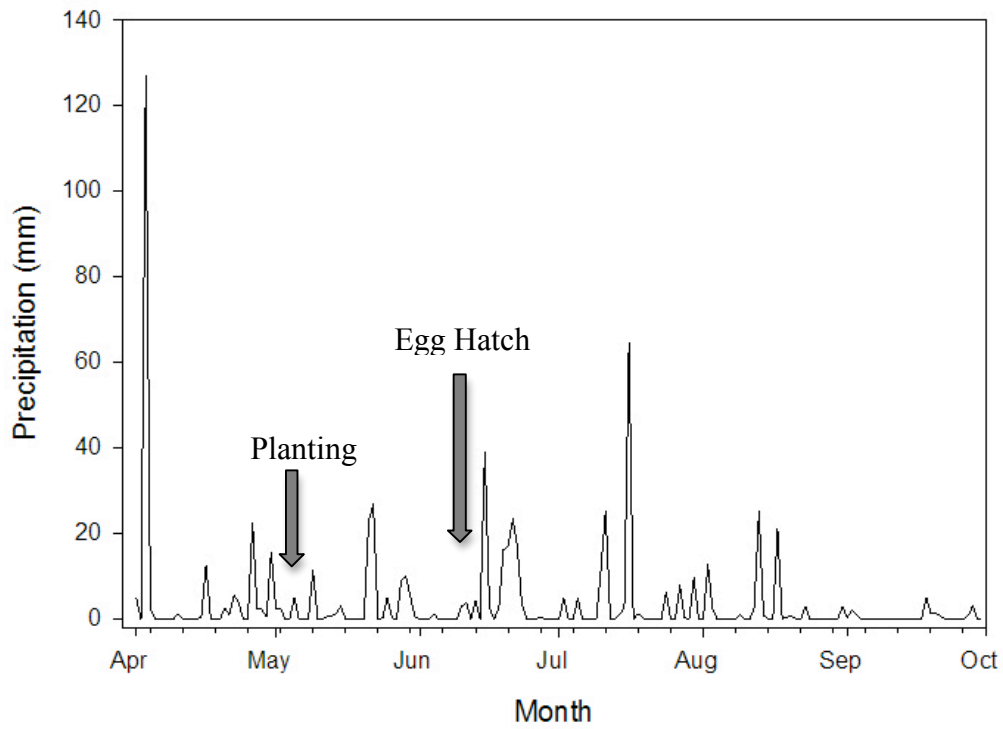


Figure 3. Monthly precipitation (mm) for the 2011 growing season at Rosemont Agricultural Research Station, MN.

Chapter III.

Nitrogen Rate Effects on Cry3Bb1 and Cry34/35Ab1 Expression in Transgenic Corn Roots

Introduction

Transgenic hybrids, expressing a lethal endotoxin, have been commercially used for a decade to control corn rootworms (*Diabrotica virgifera virgifera* Leconte, and *Diabrotica barberi* Smith and Lawrence). In 2009, performance problems (severe lodging and root injury) occurred in fields planted with a corn rootworm resistant transgenic hybrid (Ostlie 2010). The corn rootworms were able to cause substantial injury to the corn (*Zea mays*) roots in these fields despite the presence of the transgenic hybrids. These performance problems followed a spring where heavy rainfall occurred which was conducive to nitrogen leaching in the soil (Ostlie 2010). With nitrogen being a vital structural component to all proteins, it is possible that the lack of available nitrogen caused by the heavy rainfall caused a reduction in toxin production, resulting in the observed failure in the transgenic hybrid.

Transgenic hybrids expressing a toxin from the bacterium, *Bacillus thuringiensis* Berliner (Bt), were commercially released in 2003 producing Cry3Bb1 for the control of corn rootworms (Vaughn et al. 2005). In 2005 a new event (an event is the successful incorporation of a foreign gene into plant cells) was released, containing a modified version of the Cry3Bb1 toxin with an enhanced promoter (e35S), known as event MON88017 (Nguyen and Jehle 2009, EPA 2010b). This was followed by the release of a dual-acting protein combination in 2005 containing Cry34Ab1 and Cry35Ab1, known as event DAS-59122 (Storer et al. 2006, EPA 2010a). In 2009, the EPA approved hybrids pyramided with two corn rootworm resistant toxins, Cry3Bb1 and Cry34/35Ab1, thought to provide better protection to the plants and hinder the development of resistance to the Bt toxin by corn rootworms (EPA 2011). By 2011, 65 percent of the corn planted in the United States was Bt corn (USDA 2011).

Bt toxin is expressed in the roots of Bt hybrids and results in mortality of the rootworm larvae after ingestion. Although the toxin is present throughout the plant in low-doses during the growing season, its expression varies in different tissues (Nguyen and Jehle 2009). Expression levels are highest in young plants and newly formed tissues, and tend to decrease with age (Nguyen and Jehle 2009). Root tissue, expressing modified Cry3Bb1, ranges in expression from 129.7 – 40.3 µg/g dry weight between early vegetative growth and late reproductive stages, respectively (Nguyen and Jehle 2009).

Nitrogen deficiencies in corn can cause reductions in plant growth as well as protein synthesis (Bruns and Abel 2003). The level of nitrogen availability during early growth stages of the plant directly affects the amount of Bt protein produced (Bruns and Abel 2003). Whole-plant nitrogen levels in that study were positively correlated with the amount Bt protein, which was expressed in the form of Cry4ab. While nitrogen uptake does not differ in plants that express the European corn borer- resistant Bt toxin versus their isoline (Ma and Subedi 2005), this has not been evaluated for corn rootworm-resistant hybrids and its correlation with expression of the toxin. In 2007, Subedi and Ma (2007) found lepidopteran-active Bt hybrids accumulate 11% more nitrogen throughout the plant than their isolines at growth stage V7, during which heavy feeding is occurring by corn rootworms. Corn plants start rapid nitrogen uptake around growth stage V6 and end around growth stage R1 (Abendroth et al. 2011). The additional accumulation of nitrogen in Bt hybrids versus their non-Bt-isolines needs to be evaluated to find out whether additional traits (in pyramided hybrids) results in more nitrogen accumulation and requires higher fertilization rates. Current recommended nitrogen application rates in Minnesota for productive corn-on-corn fields ranges between 134-184.8 kg/ha (Kaiser et al. 2011), but these rates do not reflect any additional demands for optimal production of Bt toxins in the plants. This study examines the impact of nitrogen rates on the expression of corn rootworm resistant traits in transgenic corn, the resulting root protection, and its effects on corn rootworm populations. Specifically, we hoped to learn if nitrogen rates need to shift to optimize Bt trait expression and corn rootworm protection provided by different traits.

Materials and Methods

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Root Tissue Processing

The number of root nodes and root length were recorded to assure standard sampling. The numbers of nodes, in each root system, were recorded to standardize tissue sampling from the same growth stage of each plant. A root containing at least 2.5 cm of tissue below the soil line was selected, measured and removed. The distal 1.27cm of the root's tip was removed with a sterile razor blade and discarded. Four 0.32 cm cross-sections were cut from the root and placed into pre-weighed vials in a microtiter plate. The vials were stored on dry ice during the duration of plant processing in the field until they could be transported to the lab for storage at -80°C, where they remained until shipment. The remainder of the root biomass from all six plants in each plot was placed into a paper bag for drying and processing in the same manner as the above-ground tissue.

The cross-sections from this newest node of roots were sent to lab facilities of the respective companies for testing with a double antibody sandwiched enzyme-linked immunosorbant assay (ELISA), which quantified the amount of protein being expressed in the given amount of tissue. Samples sent to Monsanto (Monsanto, St. Louis, MO) and Dow AgroSciences (Dow AgroSciences, Indianapolis, IN) were quantitatively analyzed for the Cry3Bb1 and Cry34/35Ab1 protein (respectively) using a standard double antibody sandwich ELISA procedure as described in Nguyen and Jehle (2009).

Beetle Mortality measures

Adult beetle emergence was used as an indicator of Cry protein efficacy in each sub plot hybrid. Six cut-plant emergence cages (30.5cm by 76.2cm) (Chaddha et al. 1993) covering two plants were placed in each hybrid split plot (three per row in rows two and seven respectively). Cage locations were chosen based on plant spacing (~ 15cm apart) to ensure reproducible absolute sampling. Beetles were collected from cages every three to four days and counted by species.

Root Injury evaluations

Corn roots were dug in mid-August to evaluate root injury from corn rootworm feeding. Roots were selected, dug and washed using the same procedure as root tissue sampling (see above). Roots, pruned to 3.81cm or less, within each plant's root system were counted as a measure of corn rootworm injury. These counts, rather than the Nodal Injury Rating (NIR) (Oleson et al. 2005), were analyzed directly because the NIR data did not meet statistical assumptions for statistical analyses even when transformed.

Data analysis

Data were analyzed with an ANOVA in R (Team, 2011). Mean comparisons were performed using Fisher's LSD. Raw expression data for this study contained numerous values, which were below or above detectable limits in the ELISA. These data points were assigned the value of the lower or upper limit for the analysis; this substitution

presents the best available estimate but its inclusion may bias resulting means and diminish statistical significance. Pearson's product-moment correlation coefficient was used to measure the correlation between expression and other response variables in this study. Nitrogen applied, nitrogen uptake and percent nitrogen were evaluated for their correlation with protein expression due to their involvement in protein building. Biomass for above and below ground tissue was analyzed due to the potential link between it and the amount of protein production. Root injury and beetle emergence were evaluated because increased beetle emergence and root injury may indicate reduced toxin expression.

Results

Cry3Bb1 expression in corn roots differed significantly among nitrogen rates at growth stage V6 for both site 1 ($F=3.04$; $df=5,15$; $p=0.043$) and site 2 ($F= 3.34$; $df=5,15$; $p=0.032$)(Fig. 4). During growth stage V6, the highest Cry3Bb1 expression in site 1 occurred with an application rate of 224kg/ha and the lowest expression with 0kg/ha and 56kg/ha (Fig. 4). For site 2, the highest expression occurred at 280kg/ha and the lowest expression at 0kg/ha and 56kg/ha (Fig. 4). Expression of this protein did not differ at growth stage V6 between single trait hybrid (VT3) and the pyramided hybrid (SSX) at site1 or site 2 ($F=0.32$; $df=1,18$; $p=0.581$ and $F=0.32$; $df=1,17$; $p=0.581$).

Cry3Bb1 expression differed among nitrogen rates for growth stage V12 for both site 1 ($F=2.92$; $df=5,15$; $p=0.049$) and site 2 ($F= 5.16$; $df=5,15$; $p=0.0059$)(Fig. 5). The highest amount of expression occurred when 112kg/ha was applied and the lowest expression occurred when 0kg/ha and 56kg/ha was applied for site 1 at growth stage V12 (Fig. 5). For site 2, mean separation analysis showed the highest expression under 224kg/ha and the lowest expression when 0kg/ha was applied (Fig. 5). Expression due to hybrid was not significantly different at site 1 ($F=0.2811$; $df=1,17$; $p=0.603$) during growth stage V12, but was nearly significant at site 2 ($F=4.226$; $df=1,17$; $p=0.0556$) with slightly

higher expression occurring in VT Triple Pro ($\mu= 10.20$ ppm fresh weight) than in Smart Stax ($\mu=9.20$ ppm fresh weight).

Cry3Bb1 expression correlated with other response variables in this study at both growth stages (Table 4). Percent nitrogen at growth stage V6 had the highest correlation with Cry3Bb1 expression for both site 1 (0.42) and site 2 (0.53). Applied nitrogen rate had the next highest correlation coefficient with Cry3Bb1 for site 1 (0.36) and site 2 (0.52). Yield had the highest correlation to Cry3Bb1 expression at growth stage V12 for site 1 (0.34) and the second highest correlation at site 2 (0.53). Nitrogen concentration in the plant at growth stage V12 had the highest correlation with Cry3Bb1 expression for site 2 (0.55). Beetle emergence and root injury were not significantly correlated with Cry3Bb1 expression at growth stages V6 and V12 for site 1 and site 2.

Cry34Ab1 and Cry35Ab1 expression was not significantly different due to N rate at either V6 ($F=0.591$; $df=5,15$; $p=0.707$; $F=1.384$; $df=5,15$; $p=0.285$, respectively) or V12 ($F=0.850$, $df=5,15$, $p=0.536$; $F=1.349$, $df=5,15$, $p=0.297$) for site 1. Expression was not significantly different for Cry34Ab1 and Cry35Ab1 at site 2 either at growth stage V6 ($F=2.275$; $df=5,15$; $p=0.099$; $F=0.314$; $df=5,15$; $p=0.897$) or V12 ($F= 1.378$; $df=5,15$; $p=0.287$; $F=0.7739$; $df=5,15$; $p=0.583$).

Discussion

Understanding how nitrogen levels affect protein expression, resulting efficacy, and field performance is needed. Each of these events, and even their pyramid does not achieve “high-dose” status, which would normally prolong commercial life. Consequently, factors reducing nitrogen availability could reduce protein expression and potentially increase corn rootworm survival and resulting root injury. In this study, low levels of applied nitrogen significantly affected Cry3Bb1 toxin production during growth stages V6 and V12 at both sites. This is the first report of Cry toxin expression levels of Bt hybrids being significantly influenced by agronomic practices. This finding follows the

concerns voiced by agricultural professionals about the field performance failures observed in Minnesota during the 2009 growing season, where failures occurred under seasonal weather conditions that favored excessive spring nitrogen loss from heavy rainfall and flooding.

Differing expression curves found between growth stages at the two sites, used in this study, are likely due to differences between sites (Figs. 4 and 5). Both soils were characterized as Waukegan silt loam soils, but site 1 has a sand-gravel layer located ~ 61cm below the soil surface which allows for easy water drainage while site 2 is prone to flooding, which may have resulted in nitrogen leaching and reduced availability. A large rainfall occurred at these sites over a three-day period in June at corn growth stage V4, which resulted in minor flooding at site 2 (Fig. 7). The timing of this rainfall event fell on the beginning of egg hatch for the corn rootworms. Larval feeding likely began within a couple weeks after this event. The different characteristics of the sites may have resulted in variable nitrogen movement within the soil, as well as different rates of leached nitrogen following heavy rainfalls, resulting in expression differences as well as increased feeding by corn rootworm larvae.

Expression levels for this study were notably different from a previous study performed by (Nguyen and Jehle 2009). At growth stages V9 and V12, expression of Cry3Bb1 ranged from 129.7 $\mu\text{g/g}$ – 99.0 $\mu\text{g/g}$, respectively for their study while in our study expression ranged from 2.7ppm to 11.7 ppm at growth stages V6 and V12, respectively ($\mu\text{g/g}$ is equivalent to ppm). The inconsistency in expression may reflect differences in how the ELISAs were performed. Nguyen and Jehle (2009) expressed their root protein concentrations on a dry weight basis, while the Cry3Bb1 expression results shown here are based on fresh weight. Another difference is the apparent decrease in expression in corn roots they found as the season progressed, while expression in this study increased when comparing growth stage V6 vs. V12. The differences in expression as the season progressed may again be due to the use of fresh weight samples versus dry weight samples, but also the growth stages sampled for this study. Growth stage V12 is

the plant age when peak nitrogen uptake is occurring (Abendroth et al. 2011), which may allow for higher expression levels. The Cry34/35 Ab1 levels, which were shown to not be significantly affected by nitrogen, had a high degree of variability in this study. This is consistent with a picture shown by Higgins et al. (Feb. 23, 2009) where a root stain showing Cry34/35 Ab1 expression demonstrates the spatial variability along the root. The high degree of variability may have implications for resistance formation.

Correlations between expression and the other response variables collected during this study provided insights into how the corn plant responds to variable nitrogen levels. Because nitrogen is a major component of all proteins, significant correlation between Bt expression and percent nitrogen is expected, similar to the results found by Bruns and Abel (2003). The lack of a significant correlation between expression of the Cry3Bb1 toxin and beetle emergence may be due to below-ground factors such as density-dependent mortality, the method in which we collected beetle emergence data or the quality of food in these hybrids. The expression level may have never declined enough to result in increased beetle emergence, but higher nitrogen content in the plant roots increased food quality for the insect, similarly to what was found in a study by Moeser & Vidal (2004). While reduced expression did not result in an increase in beetle emergence for this study (Fig. 6), reduced expression levels in fields could result in increased tolerance by the corn rootworm to an already low-dose Bt toxin. In a study by Siegfried et al. (2005), EC_{50} (effective concentration) levels were found to be much higher than lepidopteran-active Bt expression levels in a lab study. In field situations, if CRW populations are exposed to varying expression levels of the Bt toxin, increased tolerance can be expected to occur. An increase in tolerance may contribute to Bt problems arising in fields and could result in additional cases of field-evolved resistance (Gassmann et al. 2011).

Understanding how Bt expression varies in fields under variable conditions, adds a valuable component to understanding how these transgenic crops perform and how they can be sustained for longer periods of time. Bt expression under unfavorable conditions

needs to be further explored in order to understand the extent to which environment is a factor in the expression of these traits. The conditions for the growing season in which this study took place were favorable with little flooding and no drought. Drought and flooding are two factors that may have a much larger impact on protein synthesis (Maranville and Paulsen 1972). Based on the current recommended nitrogen application rates and where optimal expression took place in this study, it does not appear that a shift in recommended nitrogen rate needs to be made. Management and monitoring nitrogen content in fields, however, will help optimize the performance and toxin expression in Bt corn hybrids.

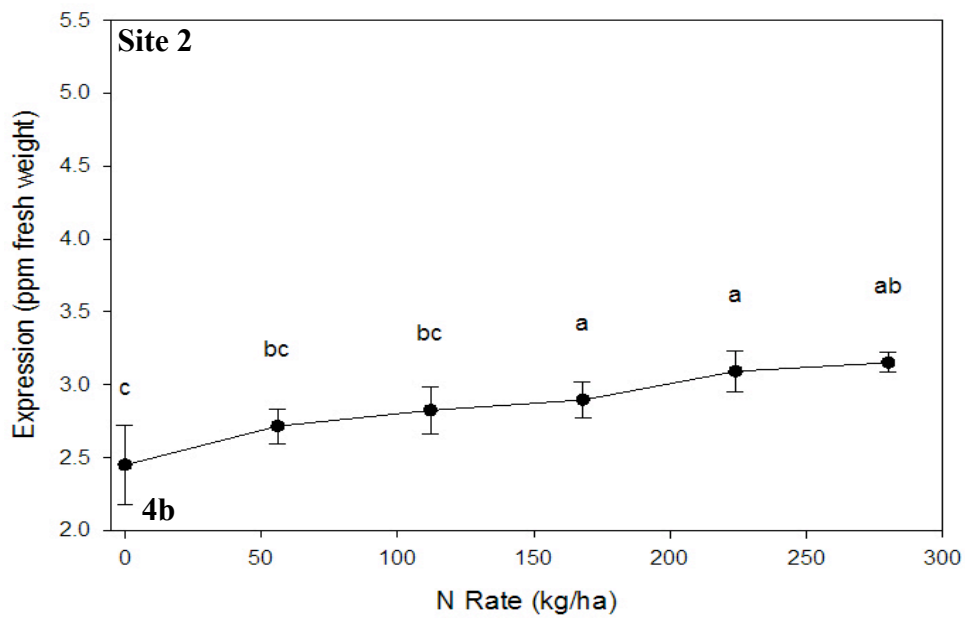
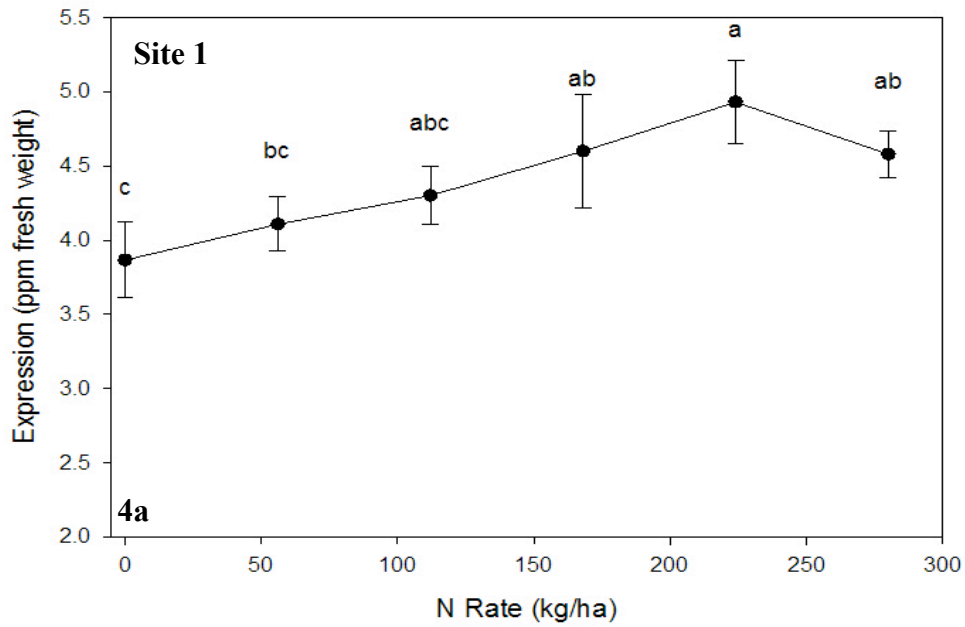


Figure 4. Expression of the Cry3Bb1 protein in root tissue during growth stage V6 in corn hybrids VT Triple and Smart Stax due to applied nitrogen rates. Fig 4a. Nitrogen rate ($p=0.043$) at site 1. Fig4b. Nitrogen rate ($p=0.032$) at site 2. Error bars represent standard errors of the means. Study conducted near Rosemont, MN 2011.

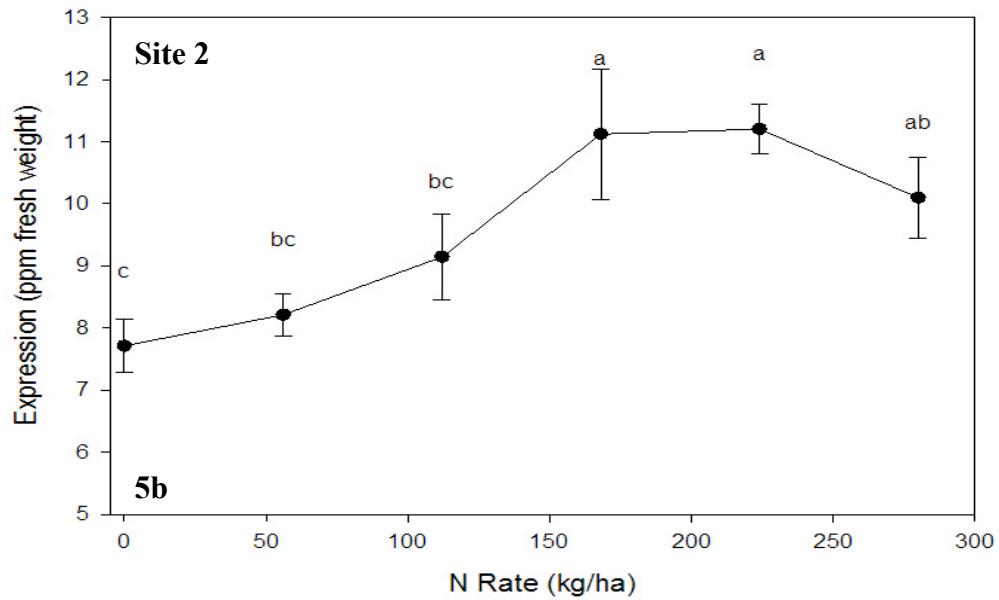
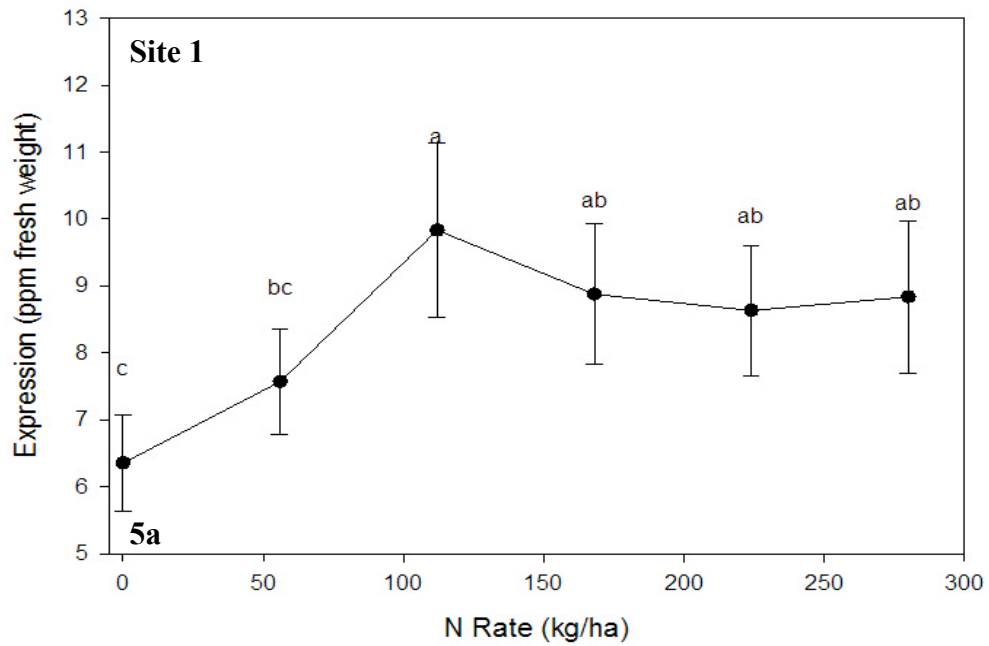


Figure 5. Expression of the Cry3Bb1 protein in root tissue during growth stage V12 in corn hybrids VT Triple and Smart Stax due to applied nitrogen rates. Figs. 5a. Nitrogen rate ($p=0.049$) at site 1. Fig. 5b. Nitrogen rate ($p=0.0059$) at site 2. Error bars represent standard errors of the means. Study conducted near Rosemont, MN 2011.

Table 4. Correlation coefficients found between various response variables and Cry3Bb1 expression from the nitrogen–Bt hybrid performance study conducted in Rosemont, MN 2011. Correlation coefficients are listed for site 1 and site 2 and then averaged together.

<u>Variables</u>	<u>Correlation Coefficients</u>		
	<u>Site 1</u>	<u>Site 2</u>	<u>Avg</u>
<u>V6 Cry3Bb</u>			
%N @ V6	0.422**	0.53***	0.48
N Rate	0.36**	0.52***	0.44
Yield	0.34*	0.44**	0.39
V6 N Uptake	0.25	0.51***	0.26
Above Biomass @			
V6	0.03	0.43**	0.22
Below Biomass @ V6	0.02	0.40**	0.20
Beetle Emergence	0.16	-0.09	0.04
Root Injury	-0.15	-0.15	-0.15
<u>V12 Cry3Bb</u>			
Yield	0.34*	0.53***	0.44
%N @ V12	0.23	0.55***	0.39
V12 N Uptake	0.31*	0.50***	0.41
N Rate	0.25	0.50***	0.38
Above Biomass @			
V12	0.28*	0.39**	0.34
Below Biomass @			
V12	0.25	0.49***	0.37
Beetle Emergence	-0.02	0.08	0.03
Root Injury	-0.14	-0.14	-0.14
			= Not Significant

- * Significance level of <0.05
- ** Significance level of < 0.01
- *** Significance level of <0.001

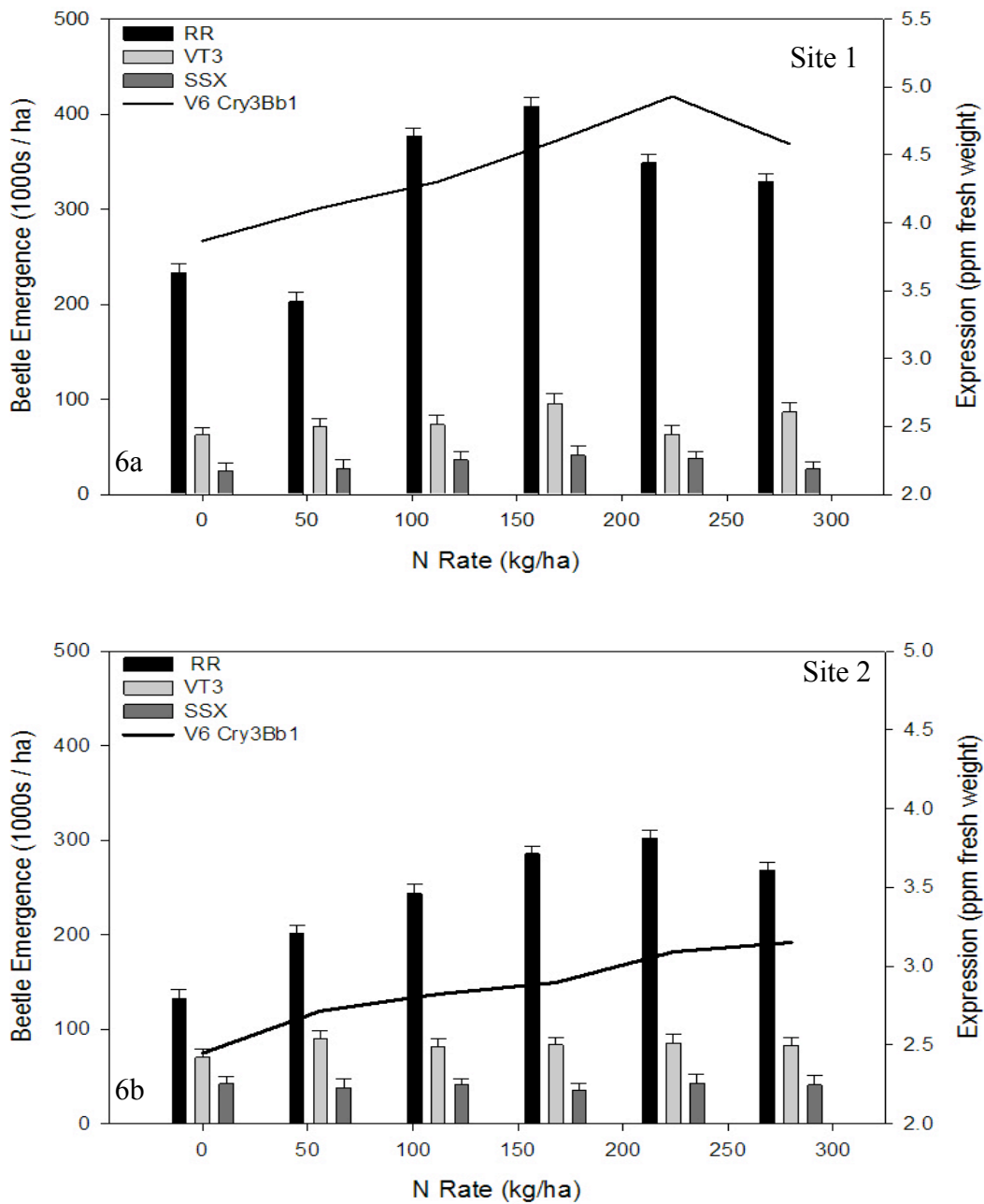


Figure 6. Beetle emergence in relation to transgenic Cry3Bb1 protein expression at growth stage V6. Fig 6a. Site 1 ($r=-0.15$). Fig. 6b. Site 2 ($r=-0.15$). Study conducted near Rosemont, MN 2011.

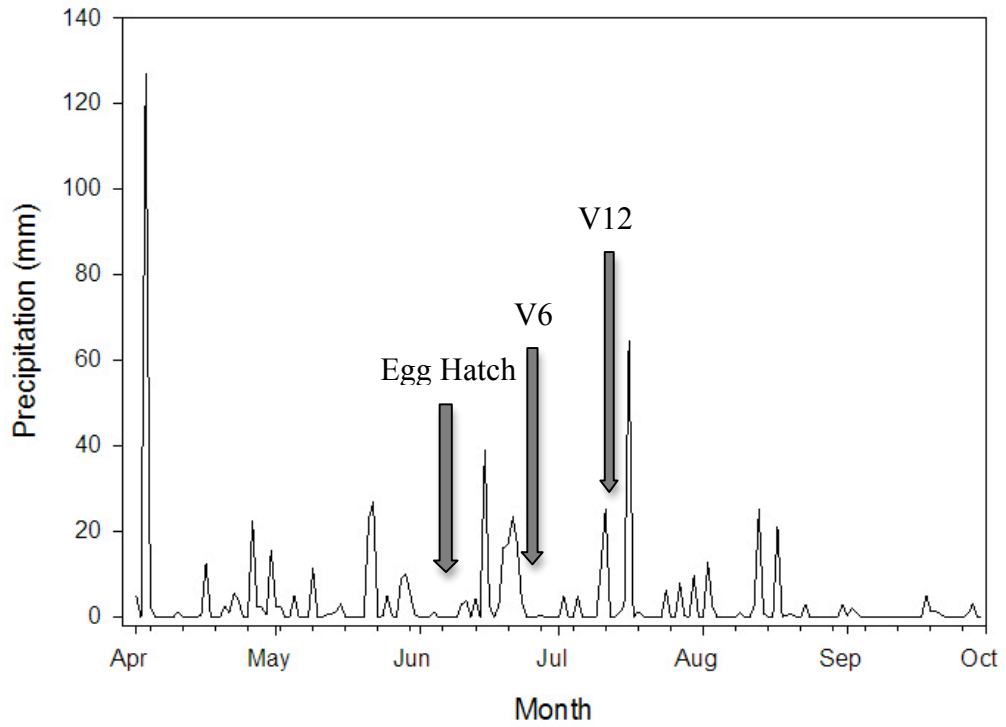


Figure 7. Monthly precipitation (mm) for the 2011 growing season at Rosemont Agricultural Research Station, MN.

Chapter IV.

Comparative Performance of Transgenic Hybrids and their Response to Nitrogen Rates

Introduction

Nitrogen rate recommendations in the U.S. have remained constant over the past 20 years for corn (*Zea mays*) while agronomic practices have been altered to increase yields. The increased demands of additional protein production required by protective traits in *Bacillus thuringiensis* Berliner (Bt) toxin-producing plants, however, has not been factored in to recommendations. Recent reports of failures of these Bt crops to control corn rootworms (*Diabrotica virgifera virgifera* Leconte and *Diabrotica barberi* Smith and Lawrence) (Gassmann et al. 2011), triggered an investigation into the influence of nitrogen on agronomic performance. Nitrogen rates applied to corn depend on several factors such as the previous crop and the nitrogen price/ crop value ratio (Kaiser et al. 2011). A previous study by (Spike and Tollefson 1991), showed that non-Bt plants were able to re-grow roots after injury with proper nutrient levels, moisture, and plant density. Root re-growth was shown to come at the expense of yield (depending on the hybrid), however, when moisture was adequate and improve yield when moisture was inadequate (Gray and Steffey 1998). Ma and Subedi (2005) indicated that non-Bt hybrids had greater nitrogen accumulation and grain yield than Lepidopteran-active Bt hybrids. Lepidopteran- active Bt hybrids were also shown to take two to three days longer to reach maturity than non-Bt hybrids. These previous studies coupled with reports of recent failures of Bt hybrids suggest a need for an performance comparison among the hybrids. Current recommended nitrogen application rates for productive corn-on-corn fields in Minnesota range between 134-184.8 kg N per ha⁻¹ (Kaiser et al. 2011), but do not account for any additional demands put forth by transgenic hybrids to produce Bt Cry proteins.

Corn rootworms are beetles that injure corn plants by feeding on the roots during the larval stage. Feeding injury causes significant damage in the form of nutrient up-take interference, lodging, plant stress, and secondary attack by pathogens (Kahler et al.

1985). To combat this issue, corn rootworm resistant hybrids, producing Bt toxin, have been developed. Corn rootworm larvae feed on corn roots producing the Bt toxin, which leads to mortality. Corn hybrids containing different Bt events are on the market today, which cause mortality in corn rootworm. Hybrids can contain either one Bt event (an event is the incorporation of the foreign gene into the plant cells) (an example being VT Triple [VT3]) or multiple, pyramided Bt events (an example being Smart Stax [SSX]). The Smart Stax trait package refers to eight different traits, which combat insect herbivory and provide herbicide tolerance, two of them, which protect against corn rootworm injury. Corn rootworm resistant hybrids (Bt) produce the Bt toxin throughout the growing season, but expression levels are highest in young tissue (Nguyen and Jehle 2009).

Nitrogen deficiencies in corn may cause reductions in growth as well as reductions in protein synthesis (Bruns and Abel 2003). Plant nitrogen levels are greatest in fields that are uninfested (no feeding) versus fields with significant feeding (Kahler et al. 1985). The level of nitrogen availability during early growth stages of the corn plant was shown by Bruns and Abel (2003) to have a direct affect on the amount of Bt protein production in the plant. This study examines the impact of nitrogen rates on the performance and agronomic response of transgenic hybrids and their isoline. Specifically, we hoped to learn if nitrogen rates need to shift to optimize Bt hybrid performance and yield.

Materials and Methods

Plot Design

Nitrogen and *Bt* corn effects on corn production and corn rootworms were studied in two fields at Rosemount, MN in 2011. The Waukegan silt loam (fine-silty over sandy or sandy-skeletal, mixed, supreractive, mesic, Typic Hapludoll) soil that is highly responsive to nitrogen fertilization and underlain by a sand-gravel layer that can accentuate drought stress. Fields for this study were selected based on two criteria: continuous corn production without long-term use of transgenic Bt corn and heavy,

natural rootworm pressure. The experiment featured a factorial treatment combination of nitrogen rates and Bt traits arranged in a split-plot design. Six nitrogen rates (0, 55, 112, 168, 224, 280 kg N per ha⁻¹) comprised the whole plots with three corn hybrids (DK44-60 - Round-Up™ Ready, DK44-90 -VT™ Triple Pro, and DK44-92 -Smart Stax™) (Monsanto, St. Louis, MO; Dow AgroSciences Indianapolis, IN) as the subplots. These hybrids differ in their corn rootworm protection by expressing: no rootworm resistant proteins, Cry3Bb1, and Cry3Bb1 + Cry34/35Ab1, respectively. Each treatment combination was replicated 4 times with plots containing eight rows (76.2 cm row-spacing) seeded at a rate of 85,185 seeds per hectare. A 3.05 m buffer zone was added between each main plot within a bed to accommodate nitrogen rate transitions during granular urea application with a small plot fertilizer.

Nitrogen Application

Nitrogen was broadcast in the form of Urea (46-0-0 N-P-K). Rates were chosen based on current recommendations from the University of Minnesota Extension (Kaiser et al. 2011). Additional rates above and below those recommended were chosen in order to get a full response curve. Sulfur (granular form) was added at a rate of 28 kg per ha⁻¹ to the entire study as recommended (Kaiser et al. 2011). Non-studied nutrients (P, K, and S) were applied at non-limiting rates. Study sites were disc chiseled prior to fertilizer application. Fertilizer was then incorporated into the soil with a cultivator.

Soil Testing

Soil samples were collected prior to fertilizer application from each main plot. Samples were analyzed for nitrate-N, phosphorus, pH, and organic matter (OM) at depths: 0-15, 15-30, and 30-60 cm to gain an understanding of the soil chemistry pre-fertilization and to have as a possibly explanatory variable for the results found in this study. Soil cores were taken from six locations within each main plot and split into respective depth cores. Each depth was homogenized in the field and a subsample was drawn for testing of NO₃⁻ (mg kg⁻¹) (Gelderman and Beegle 1998), phosphorus (Frank et al. 1998), potassium

(Warncke and Brown 1998), soil organic matter [loss on ignition] (Wang and Anderson 1998) and soil pH (Watson and Brown 1998). Soil samples were dried at 30 °C and passed through a 2mm sieve before being analysis. Characteristics of soils from this study are shown in Table 5.

Whole-plant Sampling

Six plants at growth stages V6 and V12 were randomly chosen from each plot (three each from rows three and six, respectively) based on a representative appearance in the interior of each plot (least 61 cm from the end of the row). Plants were removed with a round point shovel at least 20 cm from the base on the corn plant in order to gather as much fine root tissue as possible. The plants were then immediately washed off using a pressurized water sprayer to remove soil, mucilage coating the roots and other organic debris.

Biomass Analysis

Plants were measured for total height (base of plant to extended leaf tip) and then separated into above and below-ground portions by cutting with a sterile razor blade approximately 2.54 cm from the base of the plant. The above-ground biomass samples, comprising the bulked tissue of six plants per plot, were placed in paper bags for drying and processing. Biomass samples were left in a drier at 65°C for 24 – 48 hours and the dried composite samples were weighed. Above-ground tissue samples were then ground using a Willey mill (Thomas Scientific), and passed through a 2mm sieve. Total nitrogen content was measured using a Variomax CN Combustion Analyzer (Elementars America).

Beetle Mortality Measures

Adult beetle emergence was used as an indicator of survival in each sub plot. Six cut-plant emergence cages (Chaddha et al. 1993) were constructed and placed in each split plot (three per row in rows two and seven respectively). These cages cover an area that is 30.5cm (adjusted to reflect seeding rate) by 76.2cm. Cage locations were chosen based on plant spacing (~ 15cm apart) to ensure reproducible absolute sampling. Beetles were collected from cages every three to four days.

Root Injury Evaluations

Corn roots were dug in mid-August to evaluate root injury from corn rootworms. Shovels were placed a distance of approximately 20.32 cm away from the plant base and were driven straight down to reduce man-made injury to the root system. Dug root systems were washed with a pressurized water system to remove as much soil residue as possible. Roots, pruned to 3.81 cm or less, were counted for each plant's root system as a measure of corn rootworm injury. These counts, rather than the Nodal Injury Ratings (Oleson et al. 2005), were analyzed directly because the converted NIR ratings did not meet assumptions for statistical analyses even when transformed.

Harvesting

Yield data was gathered from the middle two rows of each eight-row plot. Total weight from both rows, as well as moisture at time of harvest, was taken to calculate yield on a kg ha^{-1} basis.

Data Analyses

Data was analyzed with an ANOVA in R (Team 2011). Mean comparisons were performed using Fisher's LSD. Nitrogen uptake data was calculated for this study by using the above ground biomass weights and percent nitrogen from the respective samples. Regression terms and critical levels were found using PROC NLIN in SAS (SAS Institute 2011). No transformations were required for the results presented.

Results

Nitrogen uptake at growth stage V6 was significantly affected by nitrogen rate and hybrid for site 1 ($p=2.04\text{e-}8$, and $p=0.0005$, respectively) and site 2 ($p<0.0001$ and $p=0.0096$, respectively) but an interaction between N rate and hybrid was not significant (Site 1: $p=0.064$, Site 2: $p=0.197$) (Fig. 8). Means separation for site 1 found VT3 to have the highest amount of nitrogen uptake with a critical level (CL) of 161.96 mg/kg and the hybrids RR and SSX not being significantly different from each other (CL: 187.73 and 122.83 mg/kg, respectively), while in site 2, VT3 and RR (CL: 201.85 and 233.11 mg/kg,

respectively) had the same nitrogen uptake rates, but SSX was significantly different with the lowest amount of nitrogen uptake and a critical level of 228.62 mg/kg. A significant difference among nitrogen uptake means for site 1 and 2 in response to nitrogen rate was found with 280 kg ha⁻¹ resulted in the highest nitrogen uptake while 56 and 0 kg ha⁻¹ resulted in the lowest nitrogen uptake (Fig. 8). No significant contribution was found with the three soil depths sampled before nitrogen application (Table 5).

Nitrogen uptake at growth stage V12 was significantly affected by nitrogen rate and hybrid for site 1 ($p < 0.0001$, and $p = 0.051$, respectively) and site 2 ($p < 0.0001$, and $p = 0.0472$, respectively) (Fig. 9). Mean separations at site 1 found VT3 to have the highest nitrogen uptake, which was significantly different from RR and SSX. Nitrogen uptake at site 1 for growth stage V12 had a linear response to nitrogen for the RR hybrid with maximum nitrogen rate applied being 280kg/ha and a quadratic response the hybrids VT3 and SSX (CL: 225 and 211, respectively). In Site 2 SSX yielded the highest nitrogen uptake with a critical level of 528.45 mg/kg, followed by RR and VT3 (CL: 379.55 and 314.01 mg/kg, respectively).

Below-ground biomass exhibited a significant interaction between nitrogen rate and hybrid at growth stage V6 for site 1 ($p = 0.0008$) (Fig. 10). The interaction was due to SSX not behaving the same as RR and VT3. SSX did not gain in biomass between 56 and 112 kg ha⁻¹ and experienced a much larger increase in biomass between 224 and 280 kg ha⁻¹. Below-ground biomass had a significant response to hybrid ($p < 0.0001$) and nitrogen rate ($p < 0.0001$) for site 2 at growth stage V6 but no interaction was detected (Fig. 10). Fisher's LSD showed a significant difference in means for all three hybrids in below-ground biomass for site 2 with RR having the highest biomass, followed by VT3, then SSX. At growth stage V12, below-ground biomass had a significant response to nitrogen rate ($p < 0.0001$) for site 1, but not to hybrid ($P = 0.02631$) (Fig. 11). For site 2, below-ground biomass had a significant response to nitrogen rate ($p < 0.0001$) and hybrid ($p = 0.005$) (Fig. 11) at growth stage V12.

A significant interaction between hybrid and N rate was found with respect to yield for site 1 ($p=0.013$) (Fig. 12). All three hybrids followed a quadratic trend in response to nitrogen rate, but RR experienced a decrease in yield (CL: 179.02 mg/kg) between 168 and 224 kg ha⁻¹ while the other two hybrids (VT3 and SSX) increased (CL: 237.67 and 271.37 mg/kg, respectively). SSX's yield remained constant from 112 to 168 kg ha⁻¹ while the other two hybrids experienced an increase in yield. Nitrogen rate had a significant effect on yield for site 2 ($p=0.0014$) (Fig. 12). Yield increased as nitrogen rate increased. While hybrids were not significantly different from each other at site 2, SSX did have a much lower CL of 219.90 mg/kg while RR and VT3 had CLs of 264.61 and 279.73, respectively.

Discussion

Transgenic traits have been inserted into corn for improved protection against multiple phytophagous insects while reducing the use of pesticides. The insertion of these traits may have unexpected consequences leading to reduced agronomic performance. Nitrogen rate and hybrid had significant impacts on different aspects of agronomic performance in this study depending on the location. Nitrogen uptake typically followed a quadratic response with respect to nitrogen rate, but this response is only observed in two of the three hybrids. Smart Stax did not plateau with the nitrogen rates used, but instead continued to increase in nitrogen uptake past 280 kg ha⁻¹ of nitrogen for site 2 at both growth stages. This suggests that Smart Stax had a higher potential nitrogen uptake, which may reflect the increased demands due to the amount of protein production from the insertion of the transgenic traits. While the hybrid VT3 expresses transgenic traits as well, there is an increase in the number of traits (three to eight) and therefore on the demands put forth on the plant in the SSX hybrid. Nitrogen uptake at growth stage V6 was similar among the three hybrids at site 1, but the VT3 hybrid was able to take up higher amounts of N at lower applied nitrogen rates. This shows an increased advantage in performance for the VT3 hybrid. In the study by Ma and Subedi (2005) on corn-borer transgenic hybrids and their isolines, no difference in N uptake was found which

contradicts our findings at growth stage V12. The difference in findings between the study by Ma & Subedi (2005) and this one may be due to the heavy corn rootworm pressure at our sites, which cause root damage. The root damage may have interfered with nutrient uptake in the RR hybrid, resulting in lower plateau than the transgenic hybrids. A study containing higher nitrogen rates is needed to complete SSX's nitrogen uptake curve and obtain consistent results between hybrids due to nitrogen rate.

Below-ground biomass had different responses at growth stage V6, depending on the site. The response mimicked what was found in the nitrogen uptake results, which explains SSX's lower biomass compared to the other two hybrids. Nitrogen up-take rates, occurring at V6, directly influenced biomass as shown in Figs. 10a and 10b. Corn rootworm feeding was also just beginning at growth stage V6, which implies that all below-ground biomass sizes can be attributed directly to genetics and agronomic performance. Round-up Ready's below-ground biomass was the highest of the three hybrids at growth stage V6, which is consistent with what was seen in the V6 uptake results. It can be hypothesized that while RR and VT3 had similar N uptake at growth stage V6, the additional demands for transgenic protein production reduced the amount of resources the VT3 plant was able to sink into below-ground biomass production, resulting in a lower root biomass than RR. At growth stage V12, below-ground biomass was significantly affected by nitrogen at both sites. The response to nitrogen is similar to the curvilinear response at growth stage V6, but all three hybrids have a similar biomass at a given nitrogen rate. The hybrids may have similar below-ground biomass at this stage due to greater corn rootworm larval feeding put on unprotected RR plants lowered the hybrids biomass.

Yield increased with increasing nitrogen availability as expected. The observed interaction between nitrogen rate and hybrid for yield at site 1 may be due to corn rootworm pressure affecting the root system (Fig. 12). The RR hybrid had more feeding damage than the other two hybrids, which may have contributed to the decrease in yield as well as a CL of only 179.02 compared to VT3 and SSX (CL: 237.67 and 271.37,

respectively). A reduction in N up-take at growth stage V6 (Fig. 8) can be seen between 168 and 224 kg ha⁻¹, which correlates to the reduction in yield for the hybrid RR and may be a contributing factor. SSX did not plateau with the nitrogen rates applied in this study at site 1. This result is likely due to nitrogen leaching after heavy rainfall (Fig. 13) since corn rootworms did not exert pressure on this hybrid like the did on the RR hybrid.

This study suggests a need for future studies examining the impact of nitrogen on transgenic hybrids, especially those containing stacked traits. The results shown could have implications for Insect Resistance Management (IRM) plans, which is a plan used to decrease the likelihood of insect resistance to the transgenic hybrids. IRM plans typically require refuge corn (non-Bt corn) to be planted within or near transgenic hybrids for the purpose of increasing random mating between the beetles emerging from refuge and transgenic hybrids. The increased biomass production and high N uptake seen in the RR hybrid (often used as the refuge hybrid) in this study may be a benefit in IRM plans. A larger root system allows for increased colonization by the beetles on the refuge hybrids, which may increase the frequency of random mating between beetles from refuge and transgenic hybrids. The hybrid SSX did not have consistent results between both sites used in this study. This suggests that variable conditions may have a large effect the performance of this hybrid.

Table 5. Soil test summary for two sites used in nitrogen rate–hybrid performance study at Rosemont, MN in 2011.

Location	Sample Depth (cm)	Soil Test				NO ₃ -N Avg
		P	K	pH	OM	
		----PPM----			%	--ppm-
Site 1	0-15	35.4	187	6.3	4.4	2.1
	15-30	----	----	----	----	2.5
	30-61	----	----	----	----	2.5
Site 2	0-15	22.3	149	6.3	4.0	1.3
	15-30	----	----	----	----	0.6
	30-61	----	----	----	----	0.2

† P, Bray-P1; K, ammonium acetate K; pH, 1:1 soil:water, OM, soil organic matter, NO₃-N, KCl extractable nitrate nitrogen.

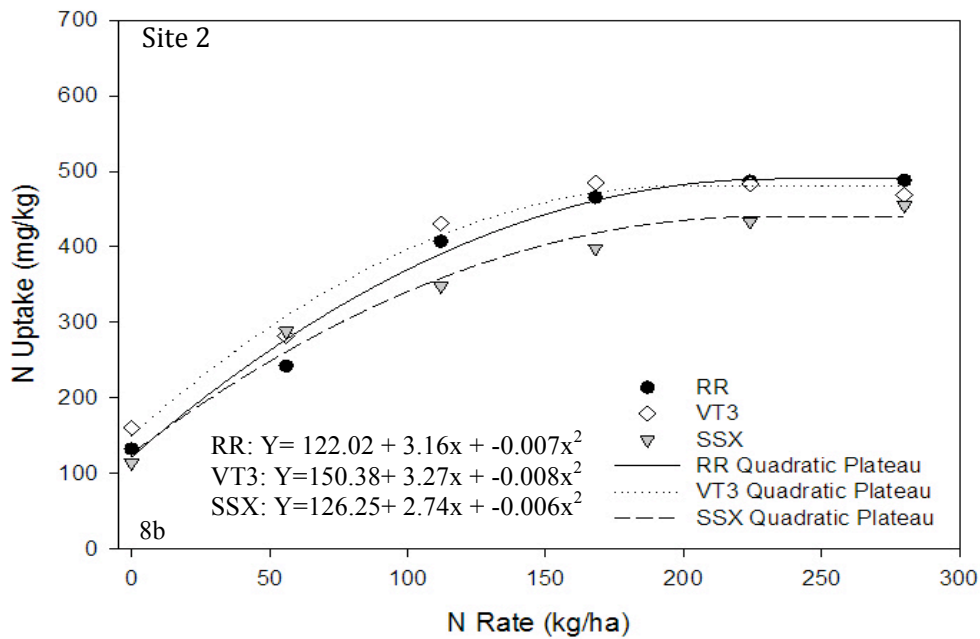
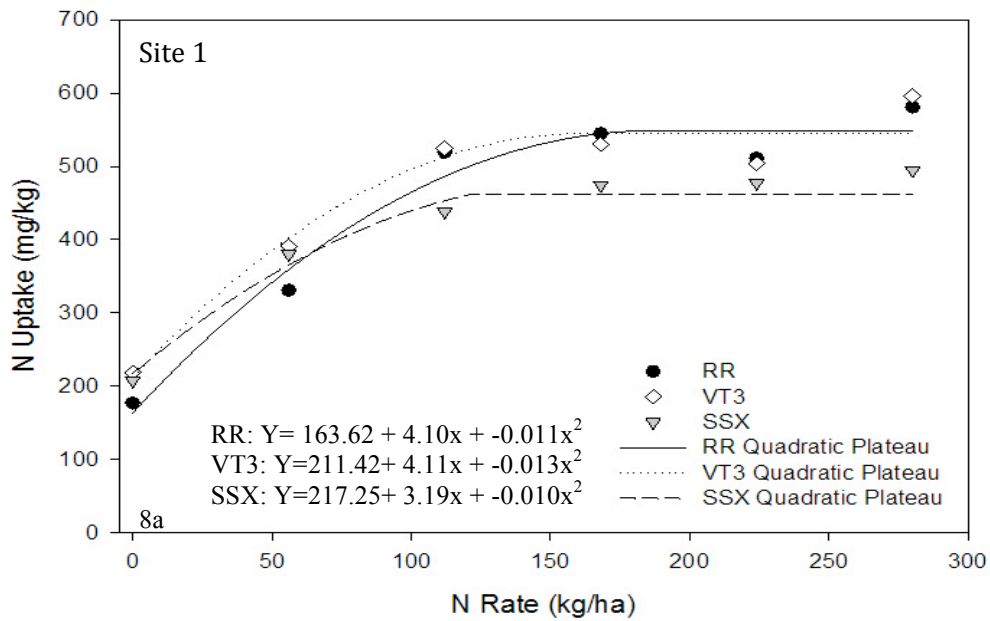


Figure 8. Nitrogen uptake in response to nitrogen rate (kg ha^{-1}) and hybrid treatments at growth stage V6. Fig. 8a. Nitrogen rate ($p < 0.0001$) and hybrid ($p = 0.0005$) at site 1. R^2 : RR=0.90, VT3=0.78, and SSX=0.79. Fig. 8b. Nitrogen rate ($p < 0.0001$) and hybrid ($p = 0.0096$) at site 2. R^2 : RR=0.88, VT3=0.74, and SSX=0.76. Nitrogen-Bt hybrid studies conducted in Rosemount, MN in 2011.

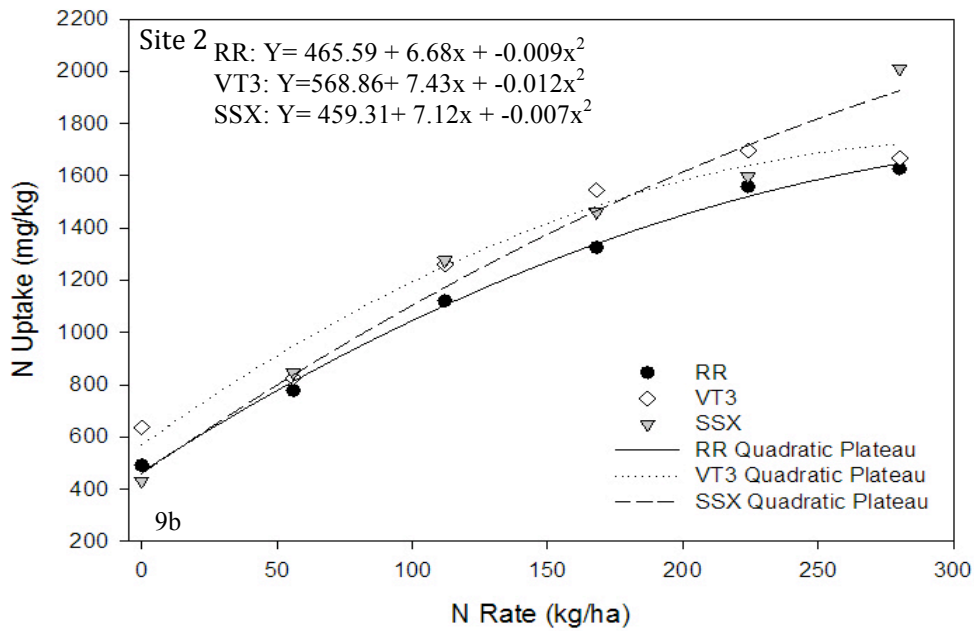
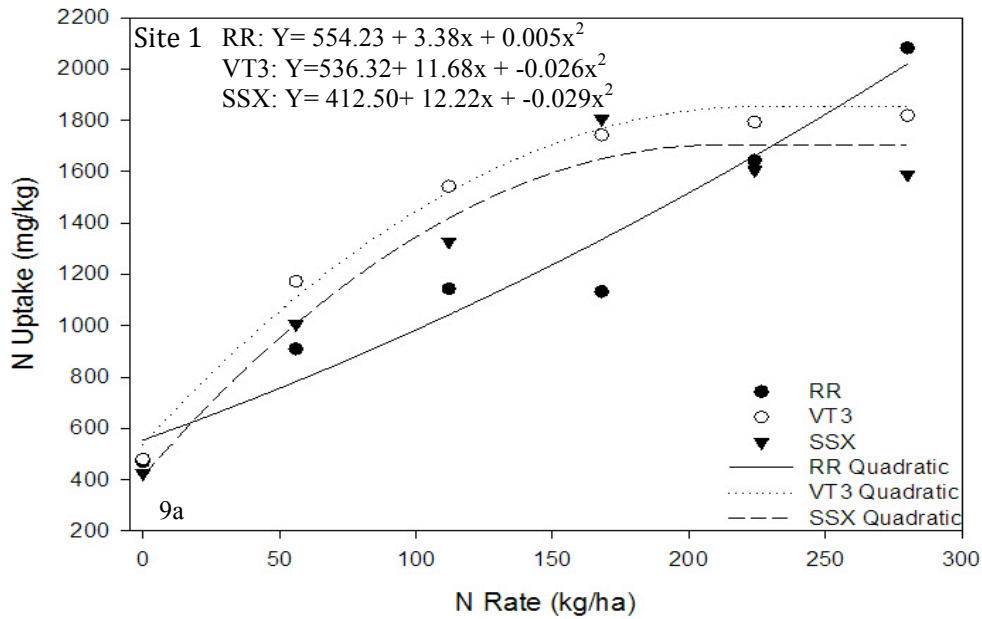


Figure 9. Nitrogen uptake in response to nitrogen rate (kg ha^{-1}) and hybrid treatments at growth stage V12 for site 1. Fig. 9a. Nitrogen rate ($p < 0.0001$) and hybrid ($p = 0.050$) at site 1. R^2 : RR=0.81, VT3=0.73, and SSX=0.79. Fig. 9b. Nitrogen rate ($p < 0.0001$) and hybrid ($p = 0.027$) at site 2. R^2 : RR=0.95, VT3=0.84, and SSX=0.80. Nitrogen-Bt hybrid studies conducted in Rosemount, MN in 2011.

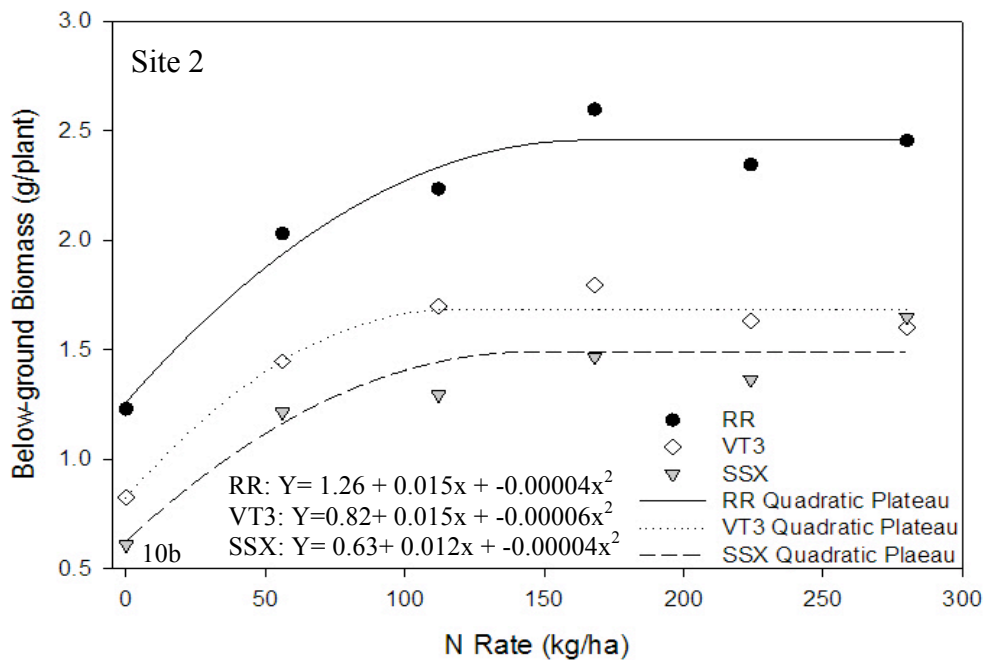
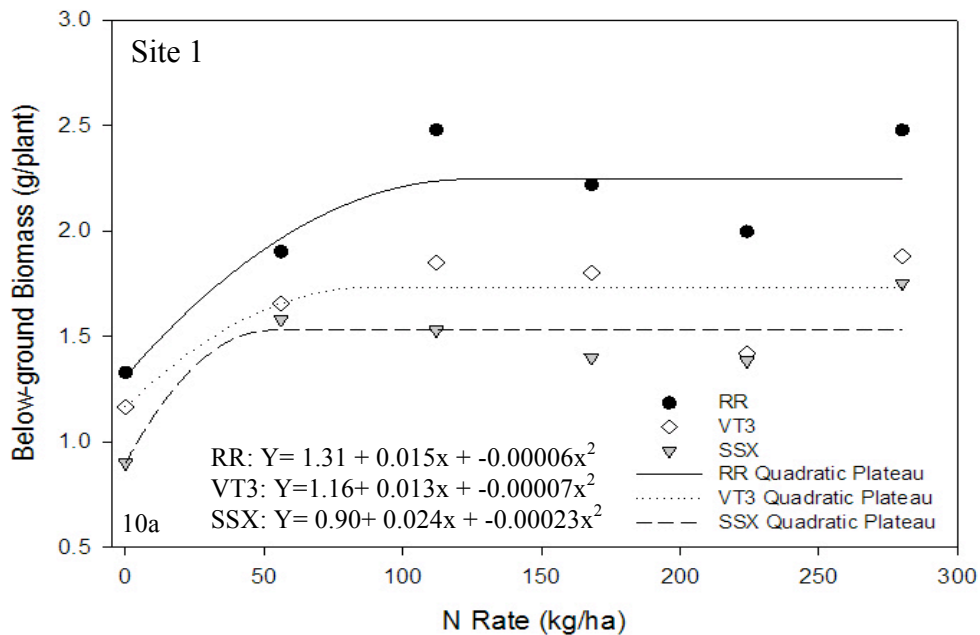


Figure 10. Below-ground biomass (g/plant) in response to nitrogen rate (kg ha⁻¹) and hybrid at growth stage V6. Fig. 10a. Nitrogen rate x hybrid (p=0.0008) at Site 1. R²:RR=0.59, VT3=0.35, and SSX=0.45. Fig. 10b. Nitrogen rate (P<0.0001) and hybrid (p<0.0001) at site 2. R²:RR=0.60, VT3=0.62, and SSX=0.78. Nitrogen-Bt hybrid studies conducted in Rosemount, MN in 2011.

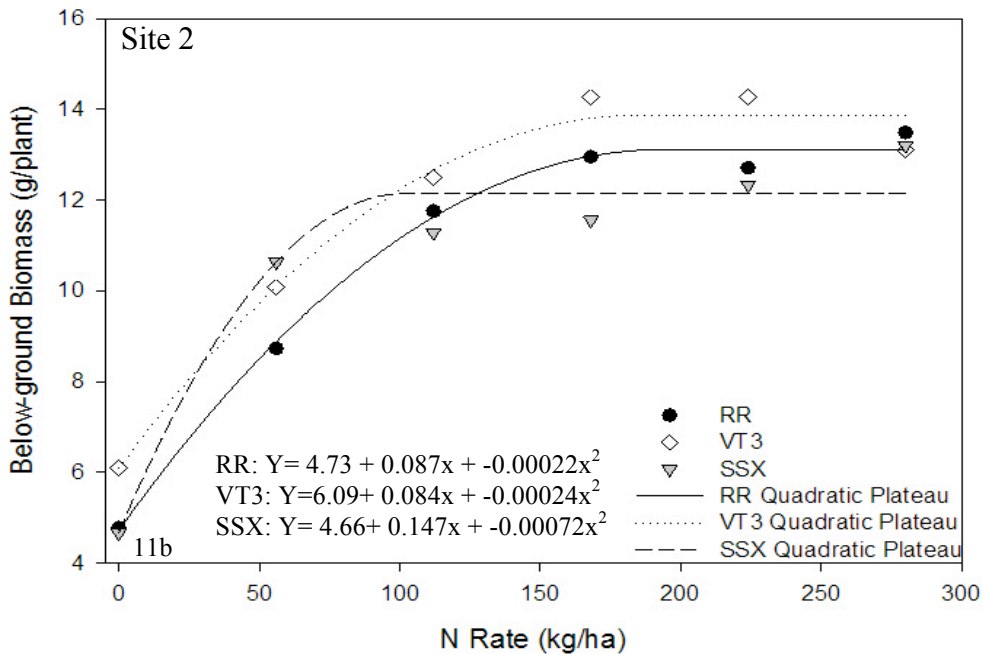
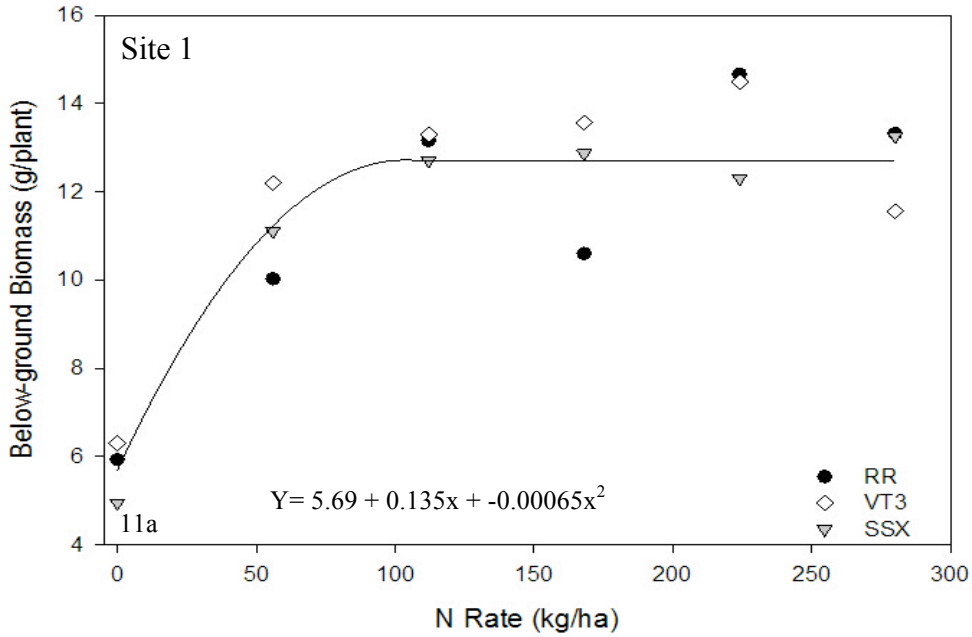


Figure 11. Below-ground biomass (g/plant) in response to nitrogen rate (kg ha⁻¹) and hybrid at growth stage V12. Fig. 11a. Nitrogen rate (P<0.0001) and hybrid (P=0.2631) at site 1. R²=0.68. Fig. 11b. Nitrogen rate (P<0.0001) and hybrid (P=0.005) at site 2. R²:RR=0.83., VT3=0.76, and SSX=0.79 . Nitrogen-Bt hybrid studies conducted in Rosemount, MN in 2011.

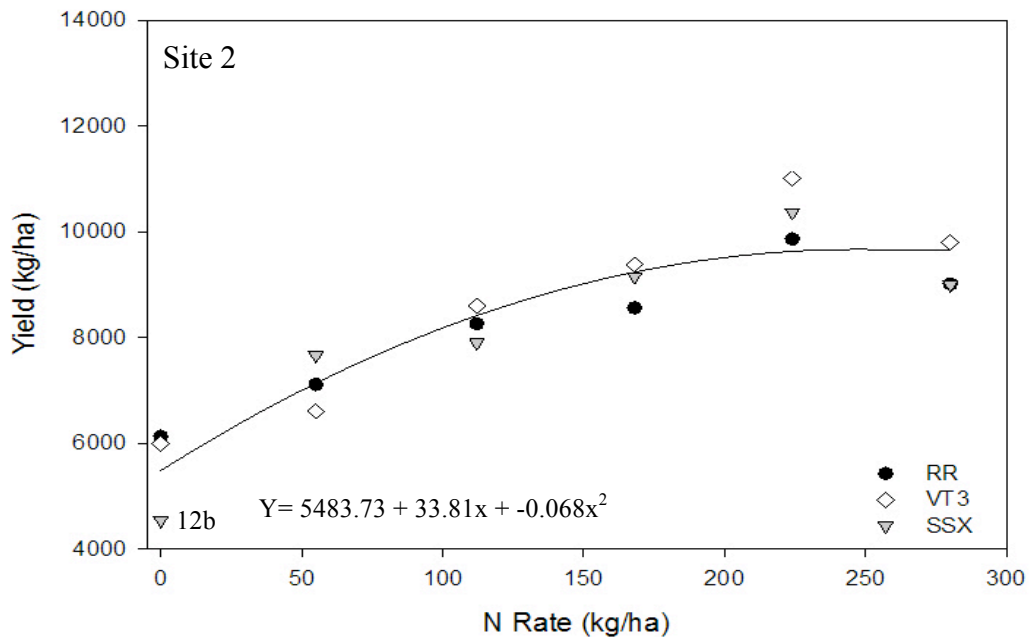
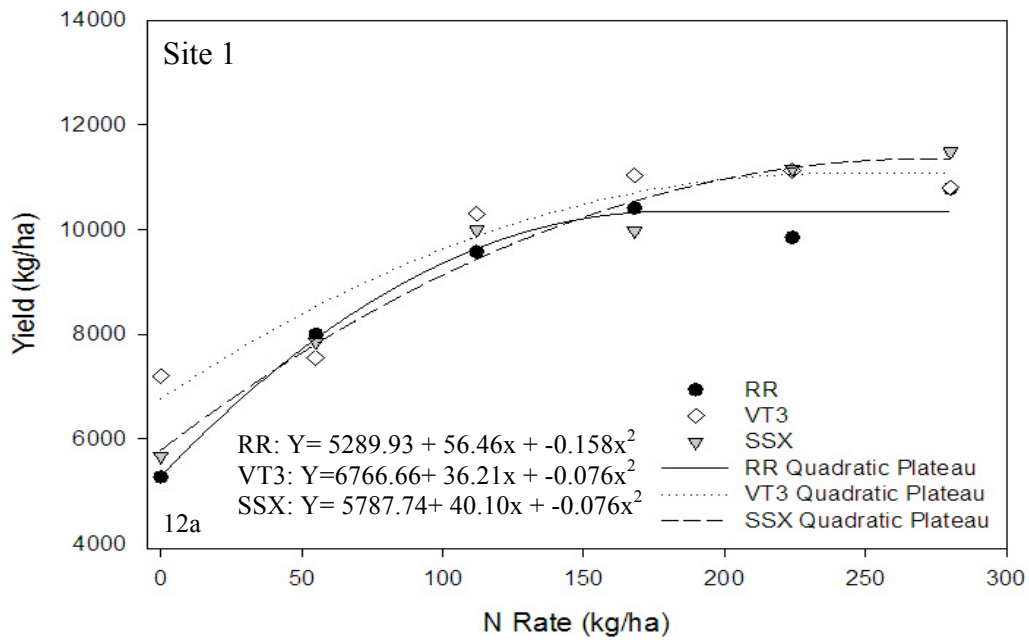


Figure 12. Yield (kg ha^{-1}) in response to nitrogen rate (kg ha^{-1}) and hybrid. Fig. 12a. Nitrogen rate x hybrid ($p=0.013$) at site 1. R^2 :RR=0.83, VT3=0.78, and SSX=0.91. Fig. 12b. Nitrogen rate ($p=0.0014$) and hybrid ($p=0.771$) at site 2. $R^2=0.51$. Nitrogen-Bt hybrid studies conducted in Rosemount, MN in 2011.

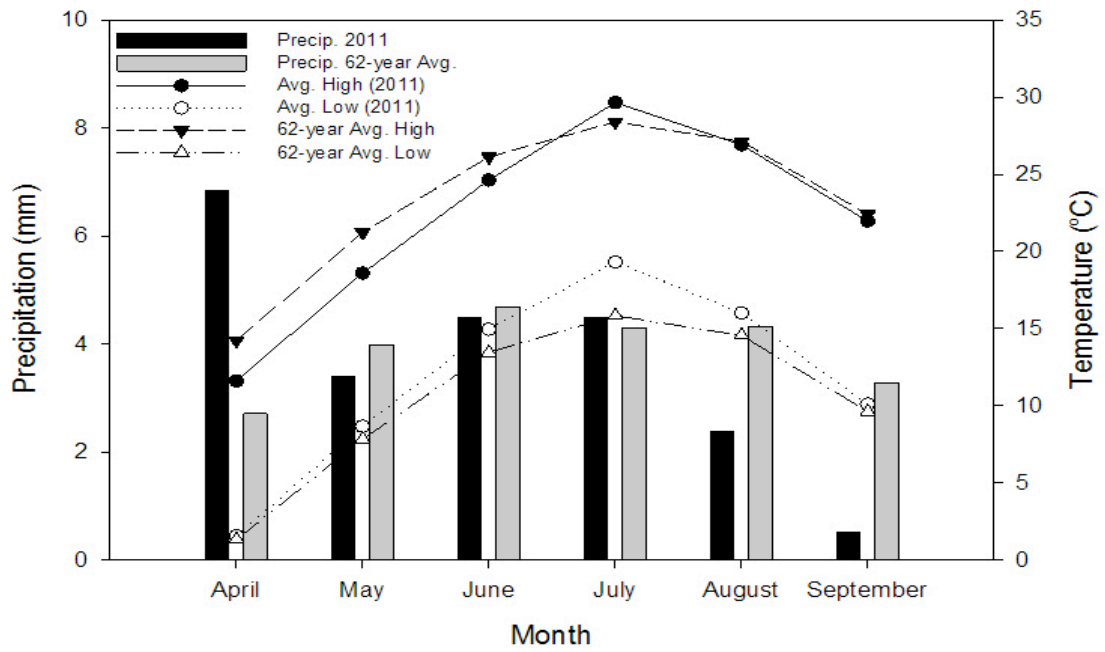


Figure 13. Temperature (°C) and rainfall (mm) averages for the 2011 growing season compared to the 62-year average at Rosemont Agricultural Research Station, MN.

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