Research Article

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The effect of nitrogen rate on transgenic corn Cry3Bb1 protein expression

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Abstract

BACKGROUND: Combining herbicide-resistant and *Bacillus thuringiensis* (*Bt*) traits in corn (*Zea mays* L.) hybrids may affect insect resistance management owing to volunteer corn. Some *Bt* toxins may be expressed at lower levels by nitrogen-deficient corn roots. Corn plants with sublethal levels of *Bt* expression could accelerate the evolution of *Bt* resistance in target insects. The present objective was to quantify the concentration of *Bt* (Cry3Bb1) in corn root tissue with varying tissue nitrogen concentrations.

RESULTS: Expression of Cry3Bb1 toxin in root tissue was highly variable, but there were no differences in the overall concentration of Cry3Bb1 expressed between roots taken from Cry3Bb1-positive volunteer and hybrid corn plants. The nitrogen rate did affect Cry3Bb1 expression in the greenhouse, less nitrogen resulted in decreased Cry3Bb1 expression, yet this result was not documented in the field.

CONCLUSION: A positive linear relationship of plant nitrogen status on Cry3Bb1 toxin expression was documented. Also, high variability in Cry3Bb1 expression is potentially problematic from an insect resistance management perspective. This variability could create a mosaic of toxin doses in the field, which does not fit into the high-dose refuge strategy and could alter predictions about the speed of evolution of resistance to Cry3Bb1 in western corn rootworm *Diabrotica virgifera virgifera* LeConte.

Keywords: volunteer corn; western corn rootworm; Cry3Bb1; *Bt*; insect resistance management; nitrogen

1 INTRODUCTION

Volunteer corn has become one of the most prevalent weed species in corn and soybean since the introduction of glyphosate-resistant soybean varieties in 1996 and glyphosate-resistant corn hybrids in 1998.[1,2] The increased presence of volunteer corn in agricultural fields has been correlated with the adoption of herbicide-resistant (HR) corn.[3] In 2012, more than 93% of the soybean and 73% of the corn planted in the United States expressed some form of HR.[4] When left unmanaged, volunteer corn can reduce the yield of both corn and soybean.[5–7] With the adoption of HR crops continuing to increase, transgenic volunteer corn will continue to be a concern for weed management in corn and soybean production. While there are chemical options to manage transgenic volunteer corn in soybean (including clethodim, diclofop, quizalofop-*p*-ethyl, fenoxaprop-*p*-ethyl, sethoxydim and fluazifop-*p*-ethyl),[7–11] managing in continuous corn is limited to row cultivation or rotation of corn hybrid genetics (i.e. planting glyphosate-resistant hybrids followed by glufosinate-resistant corn hybrids the next year).

Although the weediness of transgenic volunteer corn is a major issue, a previously uninvestigated aspect is the level of expression of insect-feeding resistance traits in volunteer plants. Currently, all transgenic insect-feeding resistance traits expressed by corn are in the form of *Bacillus thuringiensis* (*Bt*)-derived crystalline proteins. Furthermore, it is increasingly important to consider HR and *Bt* traits simultaneously when evaluating pest management in commercial corn production: in 2012, 52% of the corn planted in the United States expressed both HR and some form of *Bt* targeting above-ground lepidopteran pests (i.e. European corn borer *Ostrinia nubilalis* Hübner and others) or below-ground coleopteran pests (i.e. western corn rootworm *Diabrotica virgifera virgifera* LeConte and other *Diabrotica* species).[3]

The United States Environmental Protection Agency (USEPA) has required the planting of refuge areas where *Bt* traits are used, to delay the evolution of insect resistance to the toxins produced by *Bt* traits.[12] The refuge area of the field provides a source of insects that are not exposed to the *Bt* toxin (putatively susceptible insects). Underlying this strategy is the theory that a high-dose toxin will impose 99.99% mortality upon populations of the target pests that feed upon them.[12,13] The remaining 0.01% of insects that survive the toxin are expected to mate with susceptible insects that have developed in the refuge without exposure to the *Bt* toxin (which also comprise a majority of the insects in the

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field). High-dose refuge strategies targeting lepidopteran pests have been successful in controlling *O. nubilalis* in corn production with the *Bt* toxin Cry1Ab, as there have been no reported cases of field-evolved resistance to Cry1Ab by this pest.[14]

The high-dose refuge strategy was also implemented in 2003 when the Cry3Bb1 *Bt* toxin was first registered in corn production for control of beetles in the *Diabrotica* species complex, primarily *D. virgifera virgifera*.[15] This is in spite of the fact that, unlike Cry1Ab, Cry3Bb1 (as well as all other current commercial *Bt* toxins targeting rootworms) has never been considered to be a high-dose toxin, as it fails to meet the 99.99% mortality benchmark.[16] Prior to and since the commercialization of Cry3Bb1, multiple resistance simulation models have been published to predict the conditions that would lead to resistance to Cry3Bb1 and other in-plant toxins targeting *D. virgifera virgifera* populations.[17–21] Research also found that laboratory colonies of *D. virgifera virgifera* larvae continuously fed corn tissue expressing Cry3Bb1 had the same survival as larvae fed corn tissue that did not express the toxin after just three generations of selection.[16] In 2011, 7 years after the commercialization of Cry3Bb1, the first reported case of field-evolved resistance was documented.[22] This may be due in part to the fact that Cry3Bb1 is not a high-dose toxin, thus limiting the effectiveness of refuge strategies that are not optimized for the lower level of toxicity.[16,23]

Another potential violation of the refuge strategy is the presence of volunteer corn that expresses *Bt* traits. In one field survey, approximately 65% of transgenic volunteer corn was shown to express the Cry3Bb1 trait that was present in the hybrid planted in the field the year before, and none of these plants is accounted for in insect resistance management plans or current refuge structures.[24] The primary weed management strategy in glyphosate-resistant soybean (and other crops, including corn) involves the use of glyphosate applied post-emergence.[25] HR volunteer corn that expresses glyphosate-resistant traits will not be killed with post-emergence glyphosate applications in corn or soybean. The volunteer plants that survive and express Cry3Bb1 expose any *D. virgifera virgifera* larvae that feed upon them to the toxin, potentially adding unintended Cry3Bb1 selection pressure on rootworm populations.

In many areas of the United States, including the present study area in Indiana, corn is grown in an annual rotation with soybean. As a result, transgenic volunteer corn is frequently found growing in soybean fields. One of the major differences between corn and soybean production is the application of nitrogen (N) fertilizer in corn. Bacteria associated with the roots of soybean plants fix atmospheric N_2, so that supplemental N applications are not necessary in soybean production. Therefore, it was hypothesized that volunteer transgenic corn plants growing in soybean are likely to be deficient in N, which may affect Cry3Bb1 production.

Nitrogen fertilizer application to corn is essential in most production systems to increase plant growth and ultimately grain yield.[26] The advent of transgenic plants, which rely on the expression of inserted genes to alter the plant's defense against specific pests or herbicides, have made soil fertility and in-plant N partitioning more important.[27] In-plant expression and production of *Bt* toxins are sensitive to soil N levels.[28,29] Research has quantified the effect of N fertility on the expression of the *Bt* corn events MON-810 (Cry1Ab) and DBT 418 (Cry1Ac) in corn and cotton leaf tissue, and has shown a positive correlation between increasing levels of available soil N and *Bt* concentration.[28,29] The scientific literature has not addressed fertility effects on the production of toxins targeting coleopteran pests, including Cry3Bb1. If N does play a role in Cry3Bb1 expression, then N-deficient corn (for example, volunteer corn growing in soybean fields) may not produce the same amount of Cry3Bb1 as N-sufficient corn. A decrease in the expression of Cry3Bb1 (a toxin that is not high dose even in commercial hybrids) in volunteer plants could potentially provide sublethal doses of Cry3Bb1 to *D. virgifera virgifera* larvae. The objectives of the present research were to quantify the concentration of Cry3Bb1 expressed in volunteer and hybrid corn root tissue in various N fertility environments in order to assess whether this exposure route represents a significant parameter for consideration in current and future resistance management strategies.

2 METHODS

2.1 Greenhouse experiment

A greenhouse trial was conducted using two corn seed types [hybrid corn and the F_2 progeny of the hybrid corn (volunteer corn)] and five N concentrations (0, 25, 50, 100 and 200 mg N L^{-1}). DeKalb 61-19 (Monsanto Company, St Louis, MO) was used as the hybrid corn expressing glyphosate resistance, Cry3Bb1 and Cry1Ab. Corn kernels were hand harvested from field plots of Dekalb 61-19 in 2009 and 2010 (Monsanto Company) for use as volunteer corn seed. Forty 16 cm diameter pots (1 gal pot; Dillen Products Inc., Middlefield, OH) were filled with a mixture of baked montmorillonite clay (1.350 g) (Turface MVP; Profile Products LLG, Buffalo Grove, IL) and dolomitic limestone (0.65 g). To ensure that the baked clay stayed in each pot, the drainage holes of the pots were covered from the outside with two paper coffee filters (12-cup coffee filter; BUNN, Springfield, IL). Before planting, each pot was watered (1 L) from the greenhouse water source. Then, 20 pots were planted with three hybrid corn seeds in each, and 20 pots were planted with three volunteer corn seeds in each. After planting, water (150 mL) was added to each pot. The 40 pots were arranged in a randomized complete block design with four replicates per experimental run. The experiment was designed as a factorial arrangement of treatments with seed type (hybrid or volunteer) as factor 1 and N treatment as factor 2. Each replicate had a total of ten pots (five pots with hybrid corn, five pots with volunteer corn). Each treatment was replicated 4 times, and the entire experiment was run 3 times.

2.1.1 Fertilizer application and sampling procedures

After planting but prior to germination, each pot was watered (150 mL) twice each day at approximately 8:00 a.m. and approximately 5:00 p.m. After seed germination, a nutrient treatment (150 mL) was used instead of water at approximately 5:00 p.m. each day. The nutrient treatments rotated daily between the N treatments (source and rates of N solutions listed in Table 1) and a non-N fertilizer (0-37-37) (0-37-37 Water Soluble Fertilizer; Grow More Inc., Gardena, CA) solution mixed at a concentration of 135 mg L^{-1}. The pots were exposed to a 16 h photoperiod of artificial high-intensity, supplemental lighting, and day/night temperatures of 28/22 °C. When the corn reached the V1 growth stage (determined by the leaf collar method),[30] all of the corn plants were tested with qualitative immunoassay test strips (QuickStix™ Kit for Cry3B YieldGard® Rootworm Corn – AQ/AS 015; Envirologix Inc., Portland, ME) to determine whether the plant expressed Cry3Bb1. The pots were then thinned to one Cry3Bb1-positive corn plant per pot.

When the majority of the corn plants reached the V6 growth stage,[30] each plant was harvested. The shoots were separated from

Table 1. The chemical source and N rates used in the greenhouse experiment. The solutions were mixed with deionized water weekly and stored in the greenhouse in 3.785 L bottles. The different sources of N were used in order to equalize K and S application rates

N (mg L^{-1})	$(NH_4)_2SO_4$ (mg)	KNO_3 (mg)	KCl (mg)	$CaSO_4$ (mg)	CaCl (mg)
0	0	0	540	719	0
25	60	91	472	629	0
50	119	182	405	539	68
100	238	364	270	360	205
200	476	727	0	0	478

the roots at the soil surface, and the roots were rinsed with water to remove the baked clay. A single piece of root (approximately 2.5 cm long) was sampled from the second node of roots of each plant, approximately halfway between the root tip and the stalk. The root sample was then placed in a microcentrifuge tube (1.5 mL Economy Micro Tube with Snap Cap No. 89000-028; VWR, Radnor, PA) and frozen with liquid nitrogen. The samples were stored in a −80 °C freezer (VIP Series −86 °C Freezer – MDF-U52VA; SANYO North America Corporation, San Diego, CA) prior to quantifying Cry3Bb1. The remaining shoot and root tissue was dried at 38 °C for at least 7 days. After drying, the weight of the shoots and roots was recorded, and the tissue was then ground and analyzed for total Kjeldahl N.[31]

2.1.2 *Quantitative ELISA procedure*

The stored root samples, while still frozen, were individually removed from the microcentrifuge tube, weighed and ground with liquid nitrogen using a mortar and pestle (145 mL mortar and pestle – 60316/60317; CoorsTek, North Table Mountain, Golden, CO). Separate mortars and pestles were used for hybrid and volunteer corn samples, and each mortar and pestle was washed with 95% ethyl alcohol and clean paper towels (Scott® Brand 100% recycled fiber multifold paper towels – 01801; Kimberly-Clark Professional, Roswell, GA) between samples. After grinding, wash/extraction buffer (1 mL) (No. P-3563; Sigma Chemical Co., St Louis, MO) was added into the mortar, and the tissue was ground into the buffer with the pestle. A disposable pipette (7.5 mL disposable pipette No. 414004-006; VWR) was used to transfer the solution and contents of the mortar back into a microcentrifuge tube. Each tube was then stored in an ice bucket until root samples from each experimental run were processed. The samples were then placed back into the −80 °C freezer for 24 h prior to conducting the quantitative enzyme-linked immunosorbent assay (ELISA) procedure.

Prior to running the quantitative ELISA, the samples were thawed and homogenized. Owing to the high concentration of Cry3Bb1 in the samples and the detection ceiling of the ELISA protocol (30 μg Cry3Bb1 L^{-1}), a second set of microcentrifuge tubes were prepared. Wash/extraction buffer (999 μL) was added to each tube, and then the sample (1 μL) was added to each corresponding dilution tube. The tubes were then shaken using a lab-top vortex mixer (Advanced Vortex Mixer No. 14005-824; VWR) for 5 s at 1000 rpm to homogenize the solution. This protocol diluted the samples from mg L^{-1} to μg L^{-1}, which allowed Cry3Bb1 to be quantified with a sandwich ELISA kit (Qualiplate™ Kit for Cry3Bb1 Corn – AP015; Envirologix Inc.). Although the ELISA kit is listed by the manufacturer as a qualitative test for the detection of Cry3Bb1, the authors developed a standard curve of known Cry3Bb1 concentrations on each ELISA microplate, along with positive and negative controls provided by the kit.

Table 2. The volumes of Cry3Bb1 and wash/extraction buffer used to create the standard curve used in each microplate. The known concentration of Cry3Bb1 was created by adding 31.9 μL of concentrated Cry3Bb1 protein (4.7 mg mL^{-1} of Cry3Bb1) to wash/extraction buffer (5 mL). Each Cry3Bb1 concentration was mixed in a 1.5 mL microcentrifuge tube

Cry3Bb1 concentration (mg L^{-1})	Cry3Bb1 (μL)	Wash/extraction buffer (μL)
1.25	40	960
2.5	80	920
5.0	160	840
10	330	670
15	500	500
20	660	340
25	830	170
30	1000	0

The standard curve for Cry3Bb1 was created by adding concentrated Cry3Bb1 protein (31.9 μL) (4.7 mg mL^{-1} of Cry3Bb1; Monsanto Company) to wash/extraction buffer (5 mL). The dilution volumes and known concentrations of the Cry3Bb1 standard curve are listed in Table 2. Each concentration was mixed in a microcentrifuge tube. Then, the standard curve of known concentrations was diluted from mg L^{-1} to μg L^{-1} using the same dilution procedure as with the samples. Once the sample preparation was complete, the ELISA kit protocol was followed. An automated wash unit (ELx50 Microplate Strip Washer; Biotek, Winooski, VT) was used during the wash step of the protocol. The plates were read with a plate reader (UVmax Kinetic Microplate Reader; Molecular Devices, LLC, Sunnyvale, CA) set for optical density readings which had dual-wavelength capability (450 and 650 nm). A standard curve was created with the known Cry3Bb1 concentrations and the corresponding optical density readings (Fig. 1). This curve was then used to quantify the concentration of Cry3Bb1 in the root samples by using the known optical density reading from the plate reader.

2.1.3 *Data analysis*

The data were checked for normality and homogeneity of variance using PROC UNIVARIATE and transformed when necessary with the appropriate transformation as suggested by the Box-Cox procedure in SAS (SAS software, v.9.2, 2002–2008; SAS Institute Inc., Cary, NC). No interaction was present between N treatment and experimental run or seed type and experimental run, and thus the data (corn plant %N) were averaged over runs for N treatment and seed type (hybrid or volunteer corn) and modeled with quadratic curvilinear regression in SAS. The biomass, volunteer and hybrid corn root %N and the Cry3Bb1 concentration data

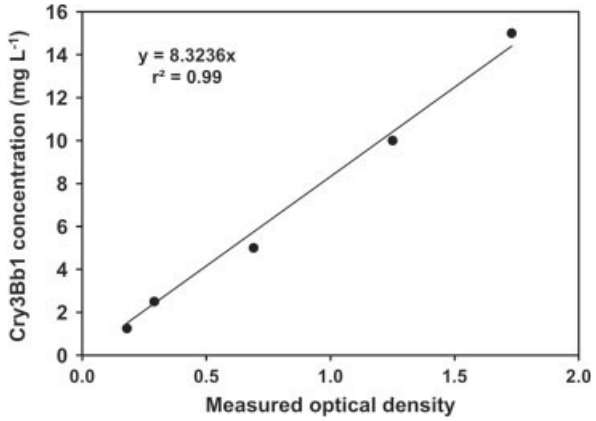

Figure 1. The Cry3Bb1 standard curve used with the ELISA protocol to quantify the concentration of Cry3Bb1 in corn roots from the greenhouse and field experiments.

were averaged over runs for N treatment and seed type (hybrid or volunteer corn) and modeled with linear regression in SAS. The slopes of regression lines were compared using analysis of covariance at $\alpha = 0.05$.

2.2 Field experiment

The field experiment consisted of growing two corn seed types [Cry3Bb1-positive hybrid corn and the F_2 progeny of the Cry3Bb1-positive hybrid corn (volunteer corn)] with five fertilizer N treatments (0, 45, 90, 180 and >300 kg N ha^{-1}). The value of >300 kg N ha^{-1} was used owing to the limit of accurate calibration of the side-dressing fertilizer unit. DeKalb 61-19 (Monsanto Company) was used as the Cry3Bb1-positive hybrid corn that expressed glyphosate resistance, Cry3Bb1 and Cry1Ab, and the F_2 of DeKalb 61-19 was used as the volunteer corn. Corn seed was hand harvested from field plots of DeKalb 61-19 in 2009 and 2010 (Monsanto Company) for use as volunteer corn. The research was conducted at two locations [Throckmorton Purdue Agricultural Center (TPAC) Lafayette, IN, and Pinney Purdue Agricultural Center (PPAC) Wanatah, IN] in 2010 and 2011 respectively. The soil type at TPAC was a Toronto-Millbrook silty loam (fine-silty, mixed, superactive, mesic Udollic Endoaqualfs) with a pH of 6.2 and 2.9% organic matter. The soil type at PPAC was a Hanna sandy loam (coarse-loamy, mixed, active, mesic Aquultic Hapludalfs) with a pH of 6.5 and 2% organic matter. Both sites were fall chisel plowed and field cultivated in the spring. The previous crop at both locations was corn. Temperature and rainfall for TPAC and PPAC are shown in Table 3. Volunteer and hybrid corn was planted in 76 cm rows at a rate of 79 000 seeds ha^{-1} at TPAC (22 April 2010) and 79 000 seeds ha^{-1} at PPAC (19 May 2011). The corn area was divided into 60 3 m wide by 9 m long plots. Experimental design was a randomized complete block design with a factorial arrangement of treatments with seed type as factor 1 and nitrogen rate as factor 2. Nitrogen as 28% urea ammonium nitrate was applied at a depth of 5–10 cm between the corn rows at the V3 growth stage[30] with a four-row side-dressing unit.

On the day that N was applied, corn leaf tissue of individual corn plants was tested with qualitative immunoassay test strips (QuickStix™ Kit for Cry3B YieldGard® Rootworm Corn – AQ/AS 015; Envirologix Inc.), and five Cry3Bb1-positive plants in each plot were flagged. When the corn reached the V6 growth stage, the five flagged plants were dug from each plot. Sampling of above-ground biomass, below-ground biomass and root pieces for ELISA testing was identical to the greenhouse procedure described above. The root samples used for Cry3Bb1 quantification were stored in a −80 °C freezer prior to determining the Cry3Bb1 expression. The remaining shoots and roots were dried at 38 °C in a drying oven for at least 7 days. After drying, the dry weight of the shoots and roots was recorded, and the tissue was then ground and analyzed for total Kjeldahl N.[31] The Cry3Bb1 concentration of the roots was determined as described in the ELISA procedure in the previous section.

Table 3. Mean monthly temperature and precipitation totals at the Throckmorton Purdue Agricultural Center (TPAC) in 2010 and the Pinney Purdue Agricultural Center (PPAC) in 2011[a]

	Temperature (°C)		Precipitation (cm)	
	TPAC	PPAC	TPAC	PPAC
Month				
April	15	9	7.4	10.4
May	18	15	6.2	11.9
June	23	21	10.6	10.4
July	24	24	6.5	13.0
August	24	21	4.4	6.1
September	20	16	2.4	8.5
Mean	20	17	—	—
Total	—	—	37.5	60.3

[a] Indiana State Climate Office—Indiana Climate Data Access Page: http://climate.agry.purdue.edu/climate/index.asp

2.2.1 *Data analysis*

The data were checked for normality and homogeneity of variance using PROC UNIVARIATE and transformed when necessary with the appropriate transformation as suggested by the Box-Cox procedure in SAS (SAS v.9.2, 2002–2008; SAS Institute Inc.). No interaction was present between year (2010 and 2011) and treatments (N rate by seed type) ($P = 0.82$). The data (corn plant and corn root %N concentration and Cry3Bb1 concentration) were pooled by seed type (hybrid or volunteer corn). There was no difference in biomass between hybrid corn and volunteer corn ($P = 0.86$), and the data were pooled. Plant biomass and corn plant %N concentration data were averaged by N treatment and seed type (hybrid or volunteer corn) and modeled with quadratic curvilinear regression in SAS. The Cry3Bb1 concentration and hybrid corn root %N concentration data were modeled with linear regression in SAS. The slopes of regression lines were compared using analysis of covariance at $\alpha = 0.05$.

3 RESULTS

3.1 Greenhouse experiment

Hybrid corn plants had more biomass than the volunteer corn plants at all N rates (Fig. 2). Increasing the N rate from 0 to 200 mg N L^{-1} resulted in increases in plant biomass for both hybrid and volunteer corn plants. Plant %N increased as higher rates of N were supplied to the plants (Fig. 3). Root %N of both hybrid and volunteer corn was also increased by increased N rate (Fig. 4). Volunteer plants had higher root %N than hybrid corn plants (Fig. 4). Cry3Bb1 concentrations were highly variable in both hybrid and volunteer corn (Fig. 5). Volunteer corn Cry3Bb1 expression was more variable than hybrid corn (the coefficient of

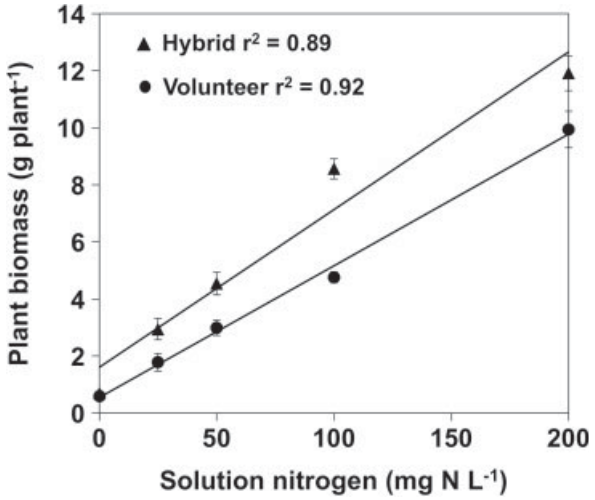

Figure 2. Hybrid and volunteer corn total biomass (shoot and root) (g plant^{-1}) in relation to the concentration of nitrogen solution supplied to greenhouse-grown plants. The data were fitted to a linear regression model, $y = \beta_0 + \beta_1 x$. Parameter estimates (± standard error) are: hybrid, $\beta_0 = 1.61 \pm 0.34$, $\beta_1 = 0.06 \pm 0.003$; volunteer, $\beta_0 = 0.55 \pm 0.24$, $\beta_1 = 0.05 \pm 0.002$. The parameter x represents the N rate. The error bars represent the standard error.

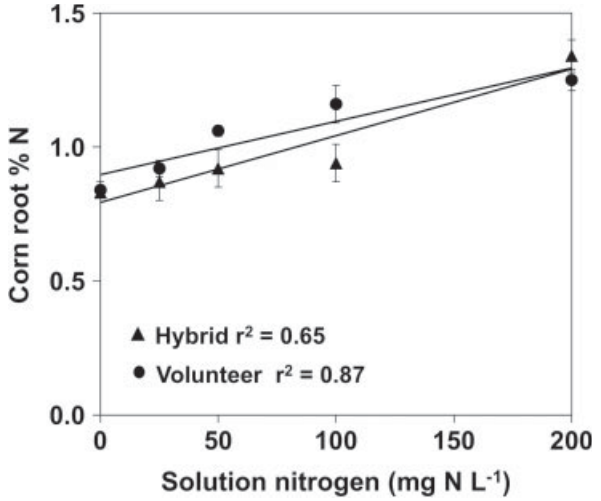

Figure 4. Hybrid and volunteer corn root nitrogen concentration (%N plant^{-1}) in relation to the rate of N supplied to greenhouse-grown plants. The volunteer data were fitted to a linear regression model, $y = \beta_0 + \beta_1 x$. Parameter estimates are: $\beta_0 = 0.90$, $\beta_1 = 0.002$. The hybrid data were fitted to a linear regression model, $y = \beta_0 + \beta_1 x$. Parameter estimates are: $\beta_0 = 0.79$, $\beta_1 = 0.003$. The parameter x represents the N rate. The error bars represent the standard error.

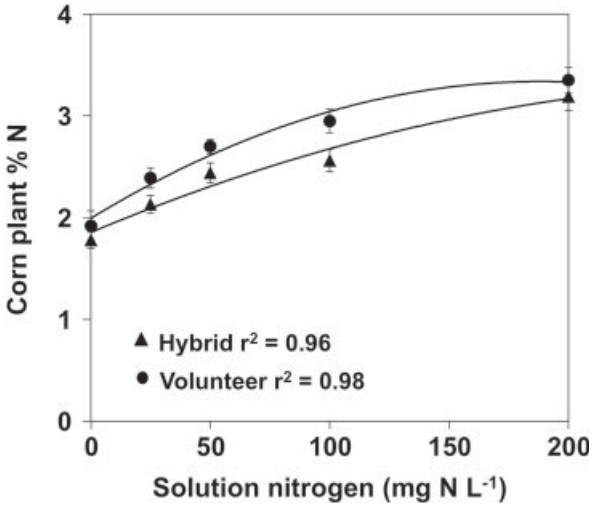

Figure 3. Hybrid and volunteer corn nitrogen concentration (%N plant^{-1}) in relation to the rate of N supplied to greenhouse-grown plants. The data were fitted to a quadratic curvilinear regression model, $y = ax^2 + bx + c$. Parameter estimates are: hybrid, $a = -2.0e^{-05}$, $b = 0.01$, $c = 1.85$; volunteer, $a = -4.0e^{-05}$, $b = 0.01$, $c = 1.99$. The parameter x represents the N rate. The error bars represent the standard error.

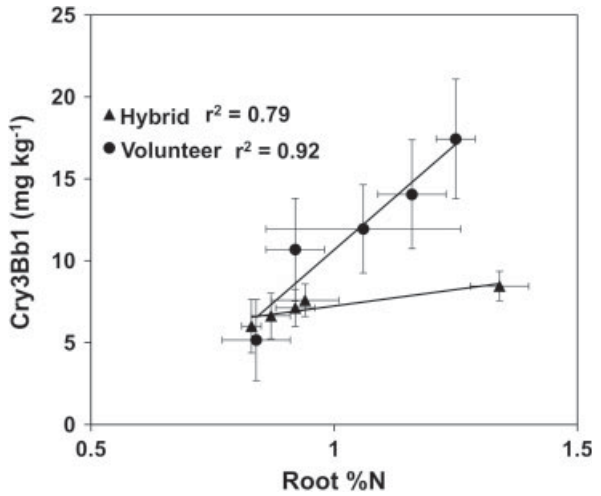

Figure 5. Hybrid and volunteer corn root Cry3Bb1 concentration (mg kg^{-1}) in relation to the %N in the root tissue of the greenhouse-grown plants. The data were fitted to a linear regression model, $y = \beta_0 + \beta_1 x$. Parameter estimates are: hybrid, $\beta_0 = 6.38 \pm 0.84$, $\beta_1 = 0.01 \pm 0.0003$; volunteer, $\beta_0 = 0.90 \pm 0.03$, $\beta_1 = 0.001 \pm 0.0003$. The parameter x represents the N rate. The error bars represent the standard error.

variation for volunteer corn was 77, and for hybrid corn 74). As the %N in the root tissue increased, the amount of Cry3Bb1 increased in volunteer corn plants, but not in hybrid corn.

3.2 Field experiment

Hybrid and volunteer corn grown in the field had equal total plant biomass at the V6 growth stage[30] (data not shown) and responded similarly to increased fertilizer N rate (Fig. 6). Total plant (Fig. 7) and root (Fig. 8) %N increased as the rate of N was increased for both hybrid and volunteer corn. Volunteer corn plants had a higher %N concentration in both plant tissues than hybrid corn plants. The field experiment was consistent with the greenhouse experiment in terms of the high variability of Cry3Bb1 expression (the coefficient of variation for volunteer corn was 108, and for hybrid corn 87), yet in the field there were no measured changes in Cry3Bb1 expression as %N in the root tissue increased in either the hybrid or volunteer corn roots (hybrid: $n = 31$, mean ± standard error = 13.1 ± 2.00, $P = 0.60$, $r^2 = 0.01$; volunteer: $n = 34$, mean ± standard error = 18.6 ± 3.41, $P = 0.72$, $r^2 = 0.005$).

4 DISCUSSION

The amount of N available for plant growth in both the greenhouse and field experiments influenced plant biomass and shoot and root N concentration. An interesting result of the greenhouse

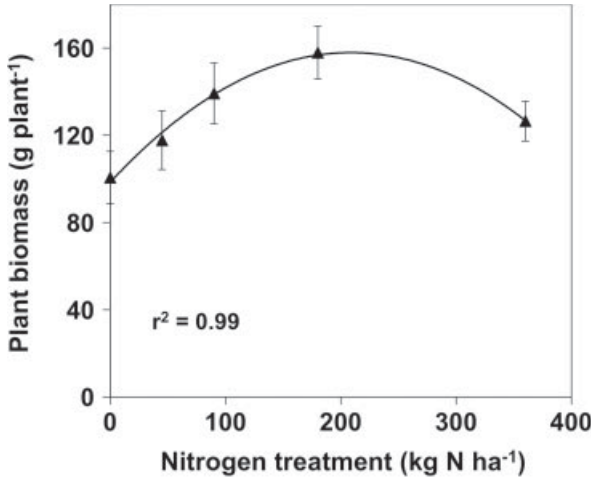

Figure 6. The pooled hybrid and volunteer corn total biomass (g plant^{-1}) in relation to the rate of nitrogen (N) applied in the field. The data were fitted to a quadratic curvilinear regression model, $y = ax^2 + bx + c$. Parameter estimates are: a = -0.00014, $b = 0.57$, $c = 0.98$. The parameter x represents the N rate. The error bars represent the standard error.

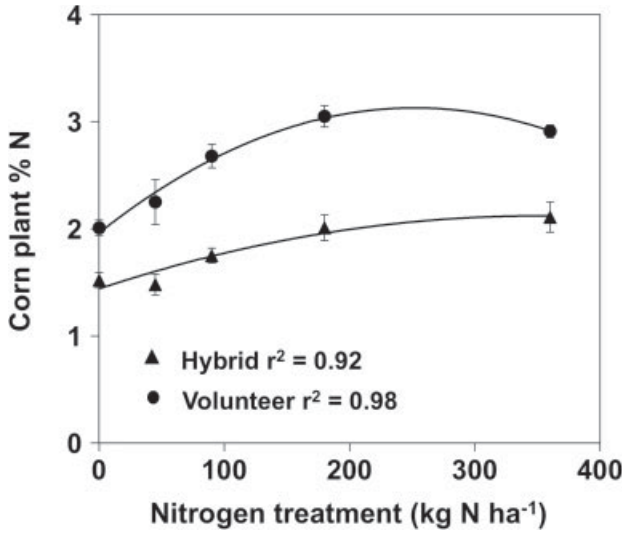

Figure 7. Hybrid and volunteer corn nitrogen concentration (%N plant^{-1}) in relation to the rate of N applied in the field. The data were fitted to a quadratic curvilinear regression model, $y = ax^2 + bx + c$. Parameter estimates are: hybrid, $a = -2.0e^{-05}$, $b = 0.009$, $c = 1.96$; volunteer, $a = -6.0e^{-06}$, $b = 0.004$, $c = 1.44$. The parameter x represents the N rate. The error bars represent the standard error.

experiment was the fact that the total biomass of the hybrid corn plants was greater than the biomass of the volunteer corn plants at each of the N rates tested (except for the 0 mg N L^{-1} treatment); however, the %N concentration in whole-plant and root tissue was greater in volunteer than in hybrid corn plants (Figs 3 and 4 and Figs 7 and 8). In the field experiment there was no difference between the biomass of the hybrid and volunteer corn plants, but, similarly to the greenhouse experiment, the volunteer plants contained higher %N in corn shoot and root tissue. In the controlled greenhouse environment, these data suggest that hybrid corn plants may be converting N into biomass more efficiently than the volunteer corn plants and clearly illustrate a difference between hybrid and volunteer corn. Volunteer plants are open-pollinated plants, allowing for slight genetic deviations

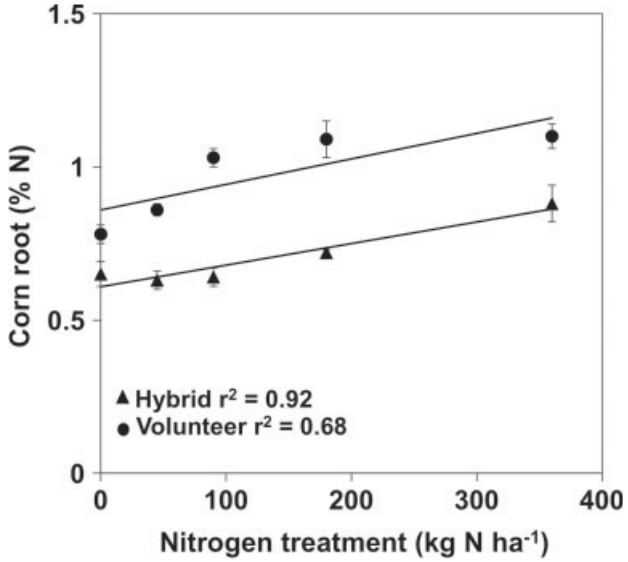

Figure 8. Field hybrid and volunteer corn root nitrogen concentration (%N plant^{-1}) in relation to the rate of N applied in the field. The volunteer data were fitted to a linear regression model, $y = \beta_0 + \beta_1 x$. Parameter estimates are: $\beta_0 = 0.86$, $\beta_1 = 0.0008$. The hybrid data were fitted to a linear regression model, $y = \beta_0 + \beta_1 x$. Parameter estimates are: $\beta_0 = 0.61$, $\beta_1 = 0.0007$. The parameter n represents the N rate. The error bars represent the standard error.

from their hybrid parents.[32] Therefore, the differences in biomass and %N between the hybrid and volunteer corn plants are not surprising. Hybrid corn often produces more biomass and yield than open-pollinated corn,[32] which is partially responsible for the average corn yield in the United States.[32]

The objective of this study was to determine the effect of N on the expression of Cry3Bb1 in hybrid and volunteer corn. It was hypothesized that, as N increased, the expression of Cry3Bb1 would also increase. The expression of Cry3Bb1 by hybrid and volunteer plants was highly variable throughout both experiments, but the controlled greenhouse experiment showed that, as the %N in root tissue increased, volunteer corn plants expressed more Cry3Bb1 (Fig. 5). Interestingly, just as with the biomass and the tissue %N data, there was a difference in the overall expression of Cry3Bb1 between hybrid corn and volunteer corn grown in the greenhouse. Previous research has indicated that N influences the expression of Cry1Ab in hybrid corn plants.[28] Unlike Cry1Ab, Cry3Bb1 expression by hybrid corn plants (unlike volunteer plants) does not appear to be affected by varying N rates. The difference in the effect of N on volunteer corn and hybrid corn Cry3Bb1 expression may be explained by the difference between the two seed types. As stated previously, open pollination of volunteer corn allows for a variety of genetic backgrounds in the volunteer seed.[32] This high level of genetic variability could result in differences in N accumulation and partitioning within individual corn plants with slightly different genetic backgrounds, resulting in expression of varying levels of Cry3Bb1.

In the field, the data did not support the hypothesis that increasing N rate would increase expression of Cry3Bb1, possibly owing to the higher amount of Cry3Bb1 variability than in the greenhouse, which is evident when comparing the coefficients of variation (greenhouse: volunteer corn 77, hybrid corn 74; field: volunteer corn 108, hybrid corn 87). The field expression of Cry3Bb1 by both volunteer and hybrid corn was more variable overall than in the greenhouse. This is a common observation in greenhouse

versus field comparisons involving plants and may be due to the differences in the biotic and abiotic conditions between the greenhouse and field. Interestingly, both greenhouse and field volunteer corn plants were more variable in Cry3Bb1 expression than the hybrid corn plants. The difference between the effect of N on Cry3Bb1 expression in the greenhouse and the field could be partially due to fact that in the greenhouse it was possible to produce a true zero N treatment. While no extra N was applied in the corresponding field treatment, field soils in the study area contained >2% organic matter and therefore did have some available N for plant growth when no artificial fertilizers were applied. Another important consideration in field studies are the hourly and daily changes in the environmental conditions to which plants were exposed in comparison with a relatively constant microclimate found in the greenhouse. The production of many plant proteins can change through a 24 h sequence owing to the circadian rhythm of the plant.[33] These temporal changes in expression have been related to environmental conditions such as photoperiod, temperature, water stress and fertility.[28,34–37] Some of the variability that was observed in the present data (especially the high variability in the field data) may be attributed to a combination of environmental factors that could have masked the N fertility effects in the field and highlights the need for caution when extrapolating greenhouse data to field situations.

While N fertility is involved in the level of expression of transgenic traits such as Cry3Bb1 in volunteer corn, fertility may not be the primary factor affecting expression of the toxin. The strict control of environmental conditions in the greenhouse may have had a larger impact on decreasing the variability in Cry3Bb1 expression, compared with the high variability in the field data. The interaction of all of the growing conditions along with the highly variable genetics of open-pollinated volunteer corn would help explain why the specific N fertility effect was not observed in the field.

5 CONCLUSIONS

The results show that there is potential for N rates to affect Cry3Bb1 expression levels. From the perspective of commercial soybean field production, the data suggest that, while the expression of Cry3Bb1 can be affected by plant available N, field soil without added N may provide the necessary building blocks for the expression of Cry3Bb1. Note that only two fields were sampled, a small subsample of the array of soil conditions and fertility levels where hybrid and volunteer corn grow. For example, there is the potential that corn grown in soil with lower levels of organic matter than the present test fields may have similar Cry3Bb1 expression as those observed in the greenhouse. From an IRM perspective, the data demonstrate that transgenic volunteer corn growing in the field can express similar levels of Cry3Bb1 as hybrid corn plants, even when the volunteer corn plants are growing in fields that have low levels of N. This result is encouraging in terms of maintaining durability of Cry3Bb1 on rootworm populations, although it must be interpreted with caution, as it has also been shown that levels of Cry3Bb1 are highly variable in both hybrid corn roots and volunteer corn roots, irrespective of the background soil fertility. This finding also has implications for the continued efficacy of Cry3Bb1 and associated IRM strategies. Corn roots that express Cry3Bb1 do not express the toxin consistently on a plant-to-plant basis and perhaps on a within-plant basis over time. On a landscape scale, this variability of toxin dose would lead to a mosaic of toxin doses both within and between plants and fields, along with a possible temporal effect. None of the current models for estimating resistance evolution in insect pests addresses this potential source of variability. In fact, one of the primary requirements of a high-dose resistance management strategy is minimizing the variation in pesticide dose.[38,39] The present data demonstrate that the presence of volunteer corn plants expressing Cry3Bb1 will increase the variability of the dose experienced by the target insects on a landscape scale.

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