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Comparison of Fumonisin Contamination Using HPLC and ELISA Methods in *Bt* and Near-Isogenic Maize Hybrids Infested with European Corn Borer or Western Bean Cutworm

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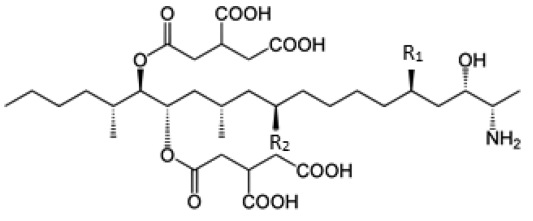
ABSTRACT: Field trials were conducted from 2007 to 2010 to compare grain fumonisin levels among non-*Bt* maize hybrids and *Bt* hybrids with transgenic protection against manual infestations of European corn borer (ECB) and Western bean cutworm (WBC). HPLC and ELISA were used to measure fumonisin levels. Results of the methods were highly correlated, but ELISA estimates were higher. *Bt* hybrids experienced less insect injury, *Fusarium* ear rot, and fumonisin contamination compared to non-*Bt* hybrids. WBC infestation increased fumonisin content compared to natural infestation in non-*Bt* and hybrids expressing Cry1Ab protein in five of eight possible comparisons; in Cry1F hybrids, WBC did not impact fumonisins. These results indicate that WBC is capable of increasing fumonisin levels in maize. Under WBC infestation, Cry1F mitigated this risk more consistently than Cry1Ab or non-*Bt* hybrids. Transgenically expressed *Bt* proteins active against multiple lepidopteran pests can provide broad, consistent reductions in the risk of fumonisin contamination.

KEYWORDS: *Bt maize, transgenic maize, GM maize, Lepidoptera, mycotoxin*

INTRODUCTION

Mycotoxigenic fungal species are worldwide contaminants of cereal grains.[1] Mycotoxins are capable of inducing detrimental health effects in humans and a variety of animals. In the Central United States, as well as in many other parts of the world, fumonisins, **1**−**3**, are the most frequently encountered mycotoxins (Figure 1). Fumonisins are produced primarily by



1 Fumonisin B_1 R_1=OH, R_2=OH

2 Fumonisin B_2 R_1=OH, R_2=H

3 Fumonisin B_3 R_1=H, R_2=OH

Figure 1. Chemical structures of fumonisin B_1, **1**; B_2, **2**; and B_3, **3**.

Fusarium verticillioides (Sacc.) Nirenberg and *Fusarium proliferatum* (Matsushima) Nirenberg, and economic losses attributable to fumonisin contamination in U.S. maize have been estimated at US$ 1−20 million under normal conditions and up to US$ 46 million in a *Fusarium* outbreak year.[2]

Fumonisin contamination in maize can be determined in a variety of ways to provide qualitative and/or quantitative data. Liquid chromatography (LC) methods provide quantitative results with the advantage of high sensitivity and specificity, but are often labor- and time-intensive and require costly equipment. Rapid test kits, including ELISA kits and lateral-flow devices, are antibody-based and can be qualitative or quantitative. Relative to HPLC methods, rapid test methods are typically less specific and their accuracy and precision are limited to a smaller range of concentrations. Because of their ease of use, relatively low cost, and faster results, rapid test kits are often preferred over more time- and labor-intensive LC methods at points of first collection in the grain handling chain. There are concerns with large-scale deployment of ELISA test kits as independent, quantitative methods. Sydenham et al.[3] found direct competitive ELISA (CD-ELISA) to measure fumonisin levels in naturally contaminated maize 2 to 3.3 times higher than HPLC, and Sutikno et al.[4] achieved a similar 2.9 times higher measurement. Indirect competitive ELISA (IC-ELISA) has compared similarly with these previous reports, with IC-ELISA obtaining higher results than HPLC for 90.6% of maize samples tested by both methods.[5] Ensuring the reliability of results obtained from rapid tests is necessary for appropriate decisions about the end use of maize grain.

Insects attacking maize ears can enhance mycotoxin contamination,[6,7] and management of these pests can provide both economic and food safety benefits, including significant reductions in fumonisin contamination. Kernel injury resulting from insect feeding contributes to fungal contamination of maize kernels. Additionally, insect larvae can initiate or exacerbate fungal infection as they act as vectors transporting fungal spores from plant surfaces to healthy or injured

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kernels.[6,8,9] Insect-injured, infected kernels are often highly contaminated with fumonisins or other mycotoxins. Kernel-feeding insects are most effectively controlled through the use of transgenic maize hybrids expressing insecticidal proteins (*Bt* maize)[7] which have been shown to have reduced fumonisin levels.[10]

Bt maize has been grown in the United States since 1996, and, as of 2013, 76% of all maize planted in the U.S. expressed some form of *Bt*-derived insect protection.[11] *Bt* maize hybrids produce insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* which confer resistance to select lepidopteran insects, but are nontoxic to humans and other nontarget species. *Bt* maize plants have an important advantage over foliar insecticides in that they produce insecticidal proteins for targeted insect control in desired plant tissues during the entire growing season. Management with foliar insecticides is costly, often requiring multiple applications that must be timed around larval hatching. Insects in the class Lepidoptera are common targets of the insecticidal proteins produced by *Bt* maize as these insects are some of the most significant pests of maize worldwide. Commercial maize hybrids that express either Cry1Ab or Cry1F proteins target European corn borer (*Ostrinia nubilalis* (Hübner)) (ECB), the most notable lepidopteran pest in maize, and are available in the U.S. and other countries.

Prior to the cultivation of *Bt* maize, annual yield losses attributable to ECB alone have been estimated near US$ 1 billion.[12] In addition to ECB control, the Cry1F protein also is effective against Western bean cutworm (*Striacosta albicosta* (Smith)) (WBC), southwestern corn borer (*Diatraea grandiosella* Dyar), fall armyworm (*Spodoptera frugiperda* (J.E. Smith)), and black cutworm (*Agrotis ipsilon* (Hufnagel)), and mildly effective against corn earworm (*Helicoverpa zea* (Boddie)) (CEW). *Bt* maize has shown high efficacy against ECB feeding, reducing overall ECB populations in areas of high *Bt* adoption.[13] This is a significant benefit to maize growers in the U.S., however, maize pests which had previously been considered secondary to ECB are now competing for this viable food source. The arrival of WBC in the Midwest U.S. in the early 1990s has posed a new threat in this maize-intensive region. Catangui and Berg[14] observed that naturally infested fields of Cry1Ab maize experienced higher WBC infestation than ECB. In a laboratory setting, WBC has displayed increased survival on Cry1Ab maize in comparison with its two primary competitors for this food source, ECB and CEW.[15] Intraguild competition for this food source is currently changing hands with WBC as a potential candidate, but minimal study has been conducted on the influence of WBC on fumonisins in various *Bt* hybrids, especially in the field. The contribution of *Bt* maize to reducing insect injury is paramount to management of *Fusarium* infection and fumonisin contamination of grain. There is a considerable lack of data with regard to impacts of WBC infestation on both *Fusarium* infection of maize and fumonisin contamination as well as the ability of various *Bt* proteins to control this species and mitigate these issues.

MATERIALS AND METHODS

Safety. Fumonisin B_1, **1**, is a group 2B carcinogen, and sodium cyanide is highly toxic. Both should be handled with appropriate caution and personal protective equipment.

Field Trials. Field experiments were conducted in Story County, IA, in 2007 (preliminary), 2008, 2009, and 2010 to assess the effects of *Bt* transformation of maize hybrids on fumonisin contamination of grain manually infested with ECB and WBC. Six commercial maize hybrids were grown; four hybrids expressed *Bt* genes which produced insecticidal proteins. Two hybrids, with maturities of 109 and 113 d, were genetically engineered to produce Cry1F insecticidal proteins (Pioneer brand hybrids 33D14 and 34A20, DuPont Pioneer, Des Moines, IA); two, with maturities of 107 and 111 d, produced Cry1Ab insecticidal proteins (DEKALB hybrids DKC57-79 and DKC61-69, Monsanto Co., St. Louis, MO); and two, with maturities of 108 and 111 d, were near-isogenic, non-*Bt* hybrids not engineered to produce any insecticidal proteins (Pioneer 34A14 and DEKALB DKC61-72). In all experiments, plots were approximately 3 m by 6.1 m and contained 4 rows. Only the middle two rows of the plot were used for insect infestation treatment and grain harvest. Plots were arranged in a randomized complete block design, and the experiment included 5 replications of each combination of maize hybrid and insect infestation treatment.

ECB infestation was performed using a volumetric dispenser to apply neonatal larvae (reared in-house) mixed with maize cob grits (Table 1).[16] All plants in the middle two rows of the assigned plots were infested. In 2007, approximately 50 neonates were applied at the base of the primary ear, near the collar, and 50 on the silks of the primary ear. In 2008, plants were infested with approximately 50 neonatal larvae on two consecutive days, the first day on the silks of the primary ear and the second day on the silks of the second-highest ear on the plant. If there was only one ear on the plant, it was infested again. In 2009 and 2010, approximately 100 neonatal larvae were applied on the primary ear, 50 at the base (near the collar) and 50 on the silks.

Table 1. Summary of 2007−2010 Field Trials

insect infestation treatments[a]	planting date	ECB infest	WBC infest	harvest date
		2007		
ECB natural WBC	May 14	July 19	July 18, 23	Oct 19
		2008		
ECB natural	May 14	July 30, 31	n/a	Oct 28
		2009		
ECB natural WBC	May 8	July 30, 31, Aug 6	July 28	Oct 30
		2010		
ECB natural WBC	April 21	July 14, 16	July 20	Oct 5

[a]Insect infestation treatment abbreviations are as follows: ECB, European corn borer (*Ostrinia nubilalis*); WBC, Western bean cutworm (*Striacosta albicosta*); natural, no insects applied.

WBC infestation methods differed by year. In 2008, three second- or third-instar larvae (hatched out of egg masses collected from naturally infested fields) were applied to the silks of each primary ear on ten ears per plot with a small painter's brush. In 2009 and 2010, egg masses on maize leaf tissue, which were collected from naturally infested fields, were attached at the base of the primary ear of 20 plants per plot, on 10 consecutive ears in each of the treatment rows. In 2009 the maize leaves were first fixed to a section of plastic screen and then attached at the base of the ear, but in 2010 the screen was not used.

Sampling. In all experiments, harvest entailed collecting ten ears per plot by hand from infested plants or, in the case of naturally infested plots, the ears were taken from the middle two rows of the plot. Ears were stored in a cold room (~4 °C) until drying to inhibit fungal metabolism and further fumonisin production. Postharvest, ears were visually assessed for insect injury and *Fusarium* ear rot severity and then dried at approximately 38 °C to below 15% moisture. In

2008 the maize was shelled prior to drying on Nov 18. In 2009 and 2010, maize was dried on-the-cob on Nov 10 and Oct 14, respectively, followed by shelling. Shelled kernels from the ten harvested ears per plot were combined and ground using a Romer Laboratories mill (Romer Laboratories, Inc., Union, MO).

Mycotoxin Analyses. *Enzyme-Linked Immunosorbent Assay (ELISA).* In 2007, fumonisin analysis was conducted by the Pioneer Hi-Bred Grain Analysis Laboratory (DuPont Pioneer, Johnston, IA) using a quantitative, competitive enzyme-linked immunosorbent assay (ELISA) with proprietary antibodies for the detection of FB_1, **1**. Samples were finely ground, and a 3 g subsample was extracted for analysis. Precoated, stabilized plates were prepared by Beacon Analytical Systems, Inc. (Saco, ME). Maize extract samples and horseradish peroxidase conjugated fumonisin B_1 were coincubated at 20−25 °C for 60−62 min with shaking in the dark. Substrate was added following washing of the plates. The substrate reaction was allowed to proceed for 30−32 min at 20−25 °C with shaking in the dark. The reaction was stopped, and the resulting color intensity of the wells was read at 450 nm. The measuring range of the fumonisin ELISA was 0.8 mg/kg to 2000 mg/kg. This method has been validated in comparison to HPLC[17] using AOAC-approved methods.[18]

In 2008 and 2009, total fumonisin concentration (FB_1 + FB_2 + FB_3), **1**−**3**, in a 10 g subsample of ground maize was determined for each plot using AgraQuant Total Fumonisin Assay 0.25/5.0 (Romer Laboratories Inc., Union, MO), a direct-competitive ELISA capable of measuring fumonisins in solution at concentrations between 0.25 and 5 mg/kg. Samples exceeding 5 mg/kg were subject to additional extract dilution to bring it within range of the ELISA test. At the time of analysis, this method was among the USDA-GIPSA performance-verified rapid test kits for the determination of total fumonisins in ground maize.

HPLC. In the preliminary experiment (2007), only the ELISA method was used. HPLC was used in 2008, 2009, and 2010.

Reagents. Fumonisin standards in 50:50 acetonitrile:water were obtained from BioPure (Tulln, Austria) and stored at 4 °C. Naphthalene 2,3-dicarboxaldehyde (NDA) was purchased from Sigma-Aldrich (Buchs, Switzerland). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ). Unless otherwise noted, 0.2 μm filtered Barnstead Nanopure deionized water (Thermo Fisher Scientific, Waltham, MA) was used in solution and sample preparation. Reagents were prepared monthly according to Bennett and Richard[19] with the modification of pH adjustment of 0.05 M phosphate buffer to 7.4 with phosphoric acid.

Sample Preparation. Sample extraction was adapted from European Standard EN 14352,[20] with modification. Briefly, 10 g of ground sample was extracted with 25 mL of methanol/acetonitrile/distilled water (25:25:50, v/v/v) by shaking on an orbital shaker for 20 min and then centrifuged at 2500*g* for 10 min. The supernatant was filtered through Fisherbrand G6 glass fiber filter paper (Thermo Fisher Scientific, Waltham, MA) and the supernatant collected in a 50 mL conical centrifuge tube. The solids were extracted in the same manner with a second 25 mL volume of extraction solution, and the collected filtrates were combined. Filtrates were stored at −23 °C prior to cleanup and analysis. Fumonisins were purified from sample extracts using Fumonistar immunoaffinity columns (Romer Laboratories, Inc., Union, MO, USA) and evaporated to dryness with a Visiprep SPE vacuum manifold (Supelco, Sigma-Aldrich, St. Louis, MO) according to package instructions.

Derivatization. Samples were derivatized with NDA reagent according Bennett and Richard[19] with some modification. Sample residues were reconstituted with 0.5 mL of methanol, followed by the sequential addition of 0.5 mL of 0.05 M sodium borate buffer, 0.25 mL of sodium cyanide reagent, and 0.25 mL of NDA reagent. Samples were heated in a 60 °C water bath for 20 min and then transferred to 4 °C for 4 min. Samples were diluted with 3.5 mL of 0.05 M phosphate buffer (pH 7.4)/acetonitrile (40:60, v/v) and transferred to 1 mL amber autosampler vials. Postderivatization, samples were stored at −23 °C for up to 24 h until transfer to the autosampler for injection, and sample analysis was completed within 2 h of this transfer.[21]

HPLC Instrumentation and Parameters. The LC system consisted of a Varian ProStar 210 pump, 410 AutoSampler (run with tray cooling at 4 °C and column oven 30 °C), and 363 fluorescence detector. The LC column was a Zorbax Eclipse Plus-C18, 3.5 μm (4.6 × 100 mm), and was preceded by a Zorbax Eclipse Plus-C18, 5 μm (4.6 × 12.5 mm), guard column (Agilent Technologies, Santa Clara, CA). Fumonisins were eluted isocratically in a mobile phase consisting of filtered deionized water/acetonitrile/glacial acetic acid (52:47:1, v/v/v) at a flow rate of 2.0 mL/min. Fumonisin−NDA derivatives were detected using 420 nm excitation and 500 nm emission wavelengths.

Method Quality Control. Limits of detection (LOD) were determined by dividing the standard deviation of the calibration curve residuals by the slope of the calibration curve at levels approaching the LOD and multiplying the result by 3.3. Limits of quantitation were calculated by multiplying 3 times the calculated LOD.[22] Method accuracy was tested by artificially contaminating duplicate ground maize samples with known amounts of fumonisin at 2 levels of contamination and testing the recoveries. Low level artificial contamination in ground maize was equivalent to 4.1, 1.2, and 0.51 μg/g FB_1, FB_2, and FB_3, respectively, and high level artificial contamination was equivalent to 6.8, 2.0, and 0.82 μg/g for FB_1, FB_2, and FB_3, respectively (Table 2). Method precision was measured by analyzing extracts of the same maize sample performed 4 times over a period of 2 weeks.

Table 2. Percent Recoveries by HPLC of FB_1, 1; FB_2, 2; and FB_3, 3; and Total Fumonisins for Low and High Spike Levels[a]

	FB_1 (%)	FB_2 (%)	FB_3 (%)	total FB recovery (%)
low spike	92.8, 104.8	81.0, 89.5	99.7, 117.6	90.9, 102.7
high spike	76.5, 85.5	71.6, 76.5	82.6, 92.2	76.0, 84.2

[a]Results are listed as repetition 1, repetition 2.

Statistical Analysis. Analysis of variance (ANOVA) was performed on log-transformed data using the PROC GLIMMIX procedure in SAS Version 9.3 software (SAS Institute Inc., Cary, NC) fitting the data to a gamma distribution. Insect feeding injury, severity of *Fusarium* ear rot symptoms, and total fumonisin concentration (FB_1 + FB_2 + FB_3) in grain were evaluated. Factorial analysis was used to determine simple effects of maize event, insect infestation treatment, and their interaction. Where interactions were present, multiple comparison analysis was performed using a Tukey−Kramer adjustment. ANOVA analysis revealed significant treatment differences among years of the study with regard to insect injury and *Fusarium* ear rot. As a result, data for these dependent effects were analyzed separately by year. Treatment effects among years of the study did not differ significantly for grain fumonisin contamination as measured by both ELISA and HPLC. The independent variable "hybrid" used for analysis combines results from each pair of hybrids used in the study which produced the same *Bt* insecticidal protein, either Cry1F, Cry1Ab, or none.

RESULTS AND DISCUSSION

Influences of Hybrid and Insect Infestation on Kernel Injury. In the 2007 preliminary study, hybrid and insect infestation significantly affected insect injury ($F_{2,77}$ = 5.93, P = 0.0040, and $F_{2,77}$ = 4.41, P = 0.0154, respectively). In 2008 and 2009, hybrid, insect treatment, and their interaction were significant factors (P < 0.0001 to P = 0.0169). In 2010, hybrid had a significant effect on insect injury ($F_{2,77}$ = 11.22, P < 0.0001). In 2007, WBC-infested plots suffered more kernel injury than either ECB (P = 0.0122) or naturally infested plots (P = 0.0123). In 2008, WBC were not available for infestations, and ECB-infested, non-*Bt* plots suffered more kernel injury (mean 6.5%) than any other hybrid/insect combination (P <

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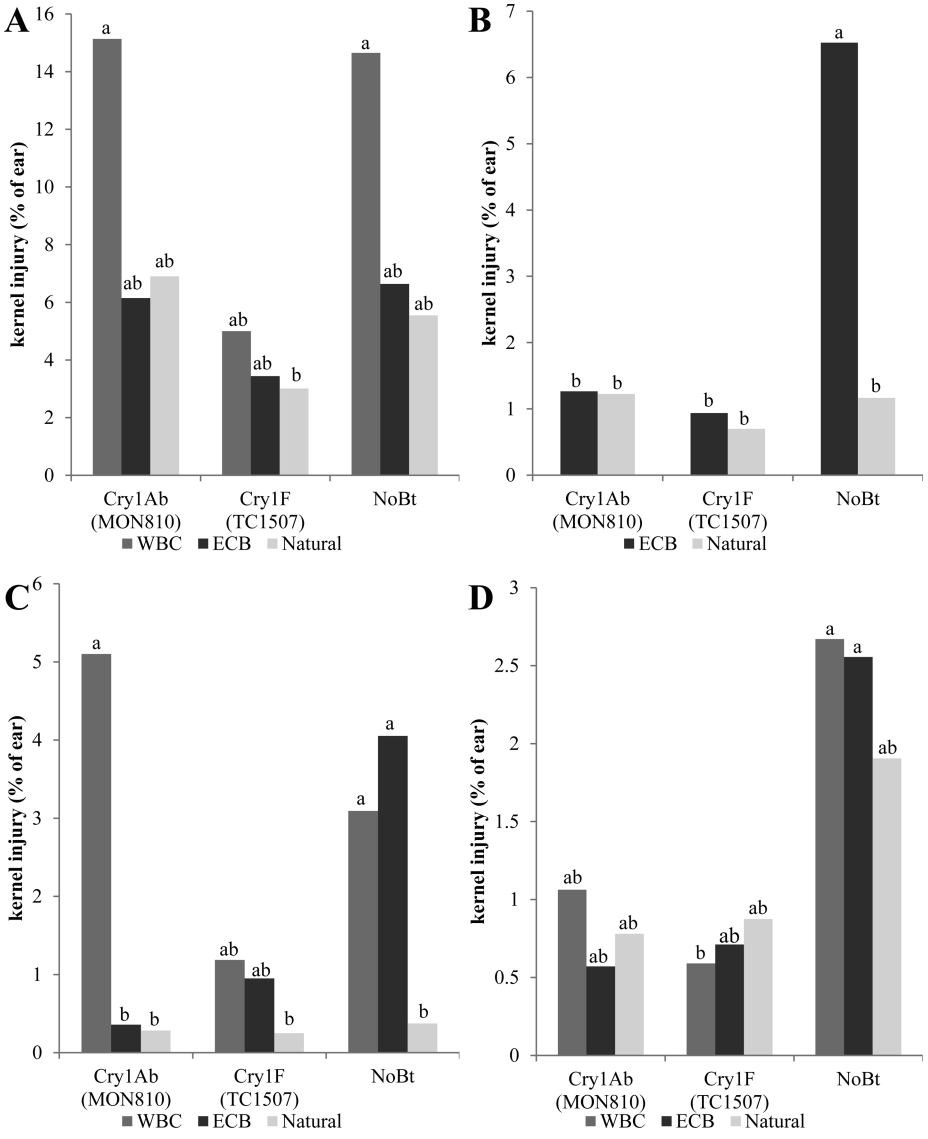

Figure 2. Insect injury (average percentage of kernels per ear) by *Bt* event for (A) 2007; (B) 2008; (C) 2009; and (D) 2010. Data are means of five replications each of two hybrids. Letters denote significant differences ($P < 0.05$) between hybrid/insect infestations within years.

0.0001 to $P = 0.0023$) (Figure 2B). In 2009, non-*Bt* hybrids infested with WBC and ECB exceeded naturally infested non-*Bt* plots ($P = 0.0035$ and $P = 0.0003$) in injury, and WBC infested Cry1Ab hybrids exceeded ECB or naturally infested Cry1Ab hybrids ($P < 0.0001$) (Figure 2C).

The susceptibility of Cry1Ab hybrids to WBC was marked in 2007 and 2009. In these years, WBC-infested Cry1Ab hybrids suffered the most kernel injury (mean 15.1% and 5.1%, respectively) (Figures 2A and 2C). Cry1F maize hybrids (*Bt* event TC1507), protected against both ECB and WBC, had very little kernel injury. In 2007, Cry1Ab hybrids and non-*Bt* hybrids experienced similar levels of kernel injury, but Cry1F hybrids suffered less injury than either Cry1Ab ($P = 0.0077$) or non-*Bt* hybrids ($P = 0.0142$). In both 2008 and 2009, non-Bt hybrids suffered more kernel injury than either Cry1Ab ($P = 0.0143$ and $P = 0.0179$) or Cry1F ($P = 0.0001$ and $P = 0.0053$) hybrids. In 2010, there were higher levels of injury in non-*Bt* as compared with either Cry1Ab ($P = 0.0005$) or Cry1F hybrids ($P = 0.0002$), and WBC incited more injury in non-*Bt* as compared to Cry1F plots (Figure 2D).

Manual insect infestations were used in this study to ensure that populations were present in the field with relatively uniform distribution. Their presence increases the likelihood of fungal infection in maize tissues affected by feeding injury and fungal spore dispersal by active larvae.[6,8] In 2007, 2008, and 2009, manual infestation significantly increased levels of kernel damage, indicating survival of manually infested insects. Insect infestations were chosen based on their traditional (ECB) and recent (WBC) significance in the growing area as well as the spectra of control provided by the *Bt* hybrids used in the study. Feeding patterns differ between ECB and WBC; WBC feeding is typically isolated at the tip of the ear and results in complete kernel destruction. ECB feeding is generally more widespread throughout the ear; the larvae leave partially intact kernels in their path as they migrate through the ear. "Railroading" is also common in ECB-infested maize, as small larvae move along a silk channel causing small amounts of injury to many kernels.[23]

Research has revealed a decline in natural ECB populations in several Midwest states as a result of the high adoption of *Bt* maize.[13] Historically, ECB has been the predominant lepidopteran pest of Midwest maize. In the years of this

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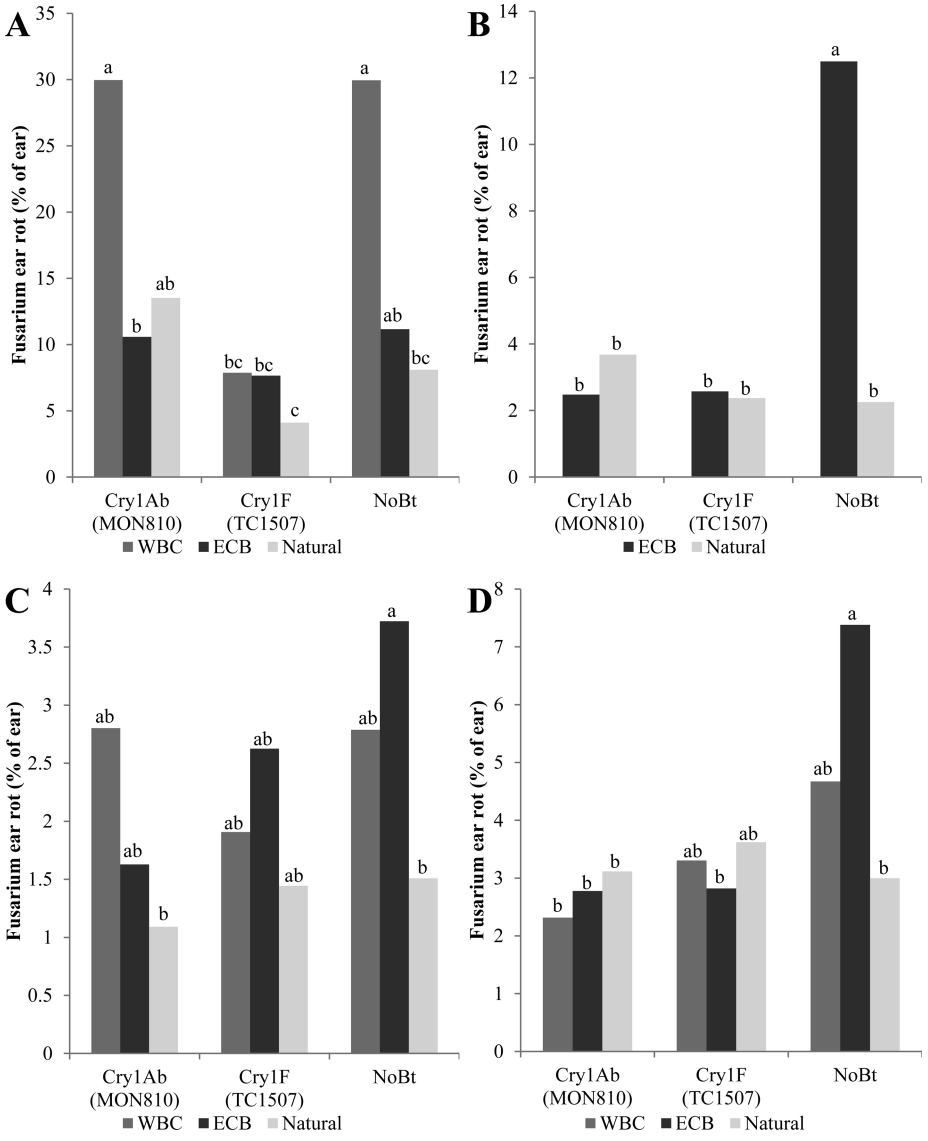

Figure 3. *Fusarium* ear rot (shown as average percent of kernels displaying visible symptoms of *Fusarium* infection per ear) by *Bt* event for (A) 2007; (B) 2008; (C) 2009; and (D) 2010. Data are means of five replications each of two hybrids. Letters denote significant differences ($P < 0.05$) between hybrid/insect infestations within years.

study, naturally infested plots did not differ in injury between susceptible and *Bt*-protected hybrids, indicating that natural ECB populations were low and injury was due to other endemic insects such as CEW. In maize-intensive regions of the U.S., regional suppression of ECB may result in predominance of pests that have historically been considered secondary to the ECB. WCB is a candidate, whose range has recently expanded from its recorded origin, Arizona,[24] all the way to Michigan, Ohio, and Pennsylvania in the U.S. as well as into Quebec.[25−27] WBC has also displayed a competitive advantage over another potential pest, CEW, when fed a diet of Cry1Ab (MON810) maize.[15] Reduced competition from ECB for maize in the Midwest will increase the need for WBC population monitoring for effective pest management, and hybrid selection will become even more important. The use of "stacked" Bt genes with different spectra of control and different modes of action may be necessary. In Puerto Rico, planting of Cry1F hybrids has been halted due to *Spodoptera frugiperda* (Lepidoptera: Noctuidae) resistance development.[28] A number of unique factors may have contributed to resistance development: little insect species migration into or off of the island; year-round maize cultivation; poor compliance with insect resistance management (IRM) requirements; and concurrent use of *Bt*-derived foliar insecticides; but this highlights the need for population monitoring and proactive resistance prevention and management in all regions where *Bt* maize is grown. Resistance has not spread to the mainland U.S.[29] and has not been noted in other lepidopteran species. In the U.S., the IRM strategy was developed to prevent resistance and, employed along with planting of *Bt* hybrids that produce different or multiple stacked *Bt* genes, can help reduce resistance development to any single gene on its own. IRM compliance is likely to improve significantly with the widespread commercialization of hybrid mixtures that include transgenic and non-transgenic (refuge) seeds in the same bag.

Influences of Hybrid and Insect Infestation on *Fusarium* Ear Rot. Hybrid and insect infestation effects on *Fusarium* ear rot were similar to those for kernel injury. Completely destroyed kernels were considered in the insect injury scores but were not physically present to contribute to

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Table 3. Total Fumonisin Concentration (FB_1 + FB_2 + FB_3), 1−3, in Maize (mg/kg) As Determined by HPLC Analysis for 2008−2010 (Mean and Standard Deviation)

	mean ± SD[a]			
insect infestation	Cry1Ab (MON810)	Cry1F (TC1507)	non-*Bt*	overall mean by year or by insect infestation within year
			2008	
	1.42 ± 1.98	**1.62 ± 2.07**	**6.60 ± 8.62**	**3.22 ± 5.69**
ECB	1.40 ± 2.31	1.99 ± 1.99	12.34 ± 9.08	5.24 ± 7.39
natural	1.45 ± 1.73	1.26 ± 2.18	0.87 ± 1.08	1.19 ± 1.68
			2009	
	0.40 ± 0.69	**0.16 ± 0.25**	**0.67 ± 1.08**	**0.40 ± 0.75**
ECB	0.30 ± 0.47	0.20 ± 0.21	1.25 ± 1.40	0.55 ± 0.91
WBC	0.78 ± 1.05	0.23 ± 0.37	0.64 ± 1.04	0.55 ± 0.87
natural	0.15 ± 0.21	0.04 ± 0.02	0.12 ± 0.15	0.11 ± 0.16
			2010	
	0.51 ± 0.52	**0.43 ± 0.66**	**0.76 ± 0.93**	**0.57 ± 0.73**
ECB	0.69 ± 0.68	0.56 ± 0.59	1.00 ± 1.27	0.75 ± 0.89
WBC	0.55 ± 0.40	0.12 ± 0.11	0.86 ± 0.79	0.51 ± 0.59
natural	0.29 ± 0.39	0.61 ± 0.94	0.42 ± 0.60	0.44 ± 0.67
hybrid means	0.70 ± 1.19	0.66 ± 1.28	2.33 ± 5.22	

[a]Means within rows are either by year (boldface type) or by insect infestation within year (lightface type). Means within columns are grouped by *Bt* insecticidal protein produced with the corresponding (*Bt* event).

Fusarium ear rot scores or fumonisin contamination; nevertheless, a strong correlation was evident between insect injury and *Fusarium* ear rot (R = 0.74, P < 0.0001). In 2007, hybrid and insect infestation treatment were both significant factors influencing *Fusarium* ear rot severity ($F_{2,77}$ = 16.46 and $F_{2,77}$ = 15.26, respectively, P < 0.0001 for both). Hybrid, insect infestation treatment, and their interaction were all significant factors in 2008 ($F_{2,50}$ = 5.48 and P = 0.0071, $F_{1,50}$ = 7.64 and P = 0.0080, $F_{2,50}$ = 7.96 and P = 0.0010, respectively). In 2009, *Fusarium* ear rot severity was influenced by insect infestation treatment ($F_{2,77}$ = 5.18, P = 0.0077), and, in 2010, it was significantly influenced by hybrid ($F_{2,77}$ = 6.43, P = 0.0026) and the interaction of hybrid and insect infestation treatment ($F_{4,77}$ = 3.69, P = 0.0084). In 2007−2009, manual insect infestations resulted in higher levels of ear rot as compared with natural (P < 0.0001 to P = 0.0213), with the exception of 2007 ECB infestation. Cry1F hybrids suffered less ear rot than Cry1Ab and non-*Bt* maize hybrids (P < 0.0001 for both) in 2007 or non-*Bt* hybrids in 2008 (P = 0.0060). Non-*Bt* hybrids subject to ECB infestation in 2008 experienced higher levels of ear rot (mean 12.5%) than any other hybrid/insect combination (P < 0.0001 to P = 0.0106) (Figure 3B). Mean ear rot levels were low among all hybrids in 2009 (2.7%, 1.8%, and 2.0% for non-*Bt*, Cry1Ab, and Cry1F hybrids, respectively) and did not differ significantly (Figure 3C). The highest level was found in ECB infested, non-*Bt* plots at 3.7%. In 2010, Cry1Ab hybrids experienced the lowest level of ear rot (mean 2.7%), which was less than that of non-*Bt* hybrids (mean 5.0%, P = 0.0019). In 2010, ECB infested non-*Bt* hybrids experienced more ear rot (mean 7.4%) than naturally infested non-*Bt* hybrids (mean 3.0%, P = 0.0072), ECB infested Cry1F hybrids (mean 2.8%, P = 0.0078), or Cry1Ab hybrids under any insect infestation treatment (P = 0.0008 to P = 0.0059) (Figure 3D).

Fumonisin Contamination. *HPLC and ELISA Method Performance.* Accurate and repeatable determination of maize fumonisin content is a crucial step in commodity management. ELISA kits are used in grain-handling industries because of their ease-of-use, rapid results, minimal sample preparation, and affordability. In this industry, overestimation of mycotoxin contamination can result in false rejections, unnecessary limitations on grain usage, and subsequent economic loss. It also restricts marketability and impacts profitability, possibly affecting maize prices and available supplies. The AgraQuant Total Fumonisin ELISA used in years 2008 and 2009 of this study has limits of detection quantitation of 0.20 mg/kg and 0.25 mg/kg, respectively. This is less sensitive than results from the HPLC method used, for which the performance characteristics follow. HPLC calibration curves for FB_1, FB_2, and FB_3 had linear regression coefficients (R) of 0.999, 0.996, and 0.998. The limits of detection for FB_1, FB_2, and FB_3 were 0.11 ng, 0.18 ng, and 0.18 ng injected, respectively, which correspond to 40 μg/kg, 65 μg/kg, and 67 μg/kg in the maize sample (at 15% grain moisture). The limits of quantitation for FB_1, FB_2, and FB_3 were 0.33 ng, 0.54 ng, and 0.54 ng, respectively, which correspond to 120 μg/kg, 195 μg/kg, 200 μg/kg in maize (at 15% grain moisture).

Recoveries for FB_1 and FB_2 were within the acceptable range established by the European Commission, but ranges for FB_3 were not established in the EC directive.[30] The mean and standard deviations for FB_1, FB_2, and FB_3 in recovery samples were 1575 ± 121, 388 ± 27, and 70 ± 1.3 ng/g, respectively. The coefficients of variation for FB_1, FB_2, and FB_3, which are representative of precision and were obtained from independent extracts of the same maize sample, were 7.7%, 7.0%, and 1.9%, respectively. For fumonisin levels determined by HPLC and samples with FB_1, FB_2, and FB_3 levels all within their respective ranges of quantitation, FB_1 constituted 69.3 ± 4.7% of the total fumonisin contamination in the grain samples (N = 24).

Occurrence of Fumonisins and Range of Concentrations in Maize Samples. ELISA determinations of fumonisin B_1, **1**, in grain from 2007 indicated a range of FB_1 sample contamination from LOD to 80.62 mg/kg with a mean of 9.98 mg/kg. Fumonisin levels were below 2 mg/kg in 19 samples, 20 samples were between 2 and 4 mg/kg, and 51 samples exceeded 4 mg/kg. Hybrid and insect infestation treatment were significant influences on FB_1 concentrations in maize ($F_{2,77}$ = 4.76, P = 0.0112, and $F_{2,77}$ = 15.70, P < 0.0001). Non-*Bt*, Cry1Ab, and Cry1F hybrids averaged 13.14 mg/kg, 11.96 mg/kg, and 4.84 mg/kg FB_1, respectively. ELISA determi-

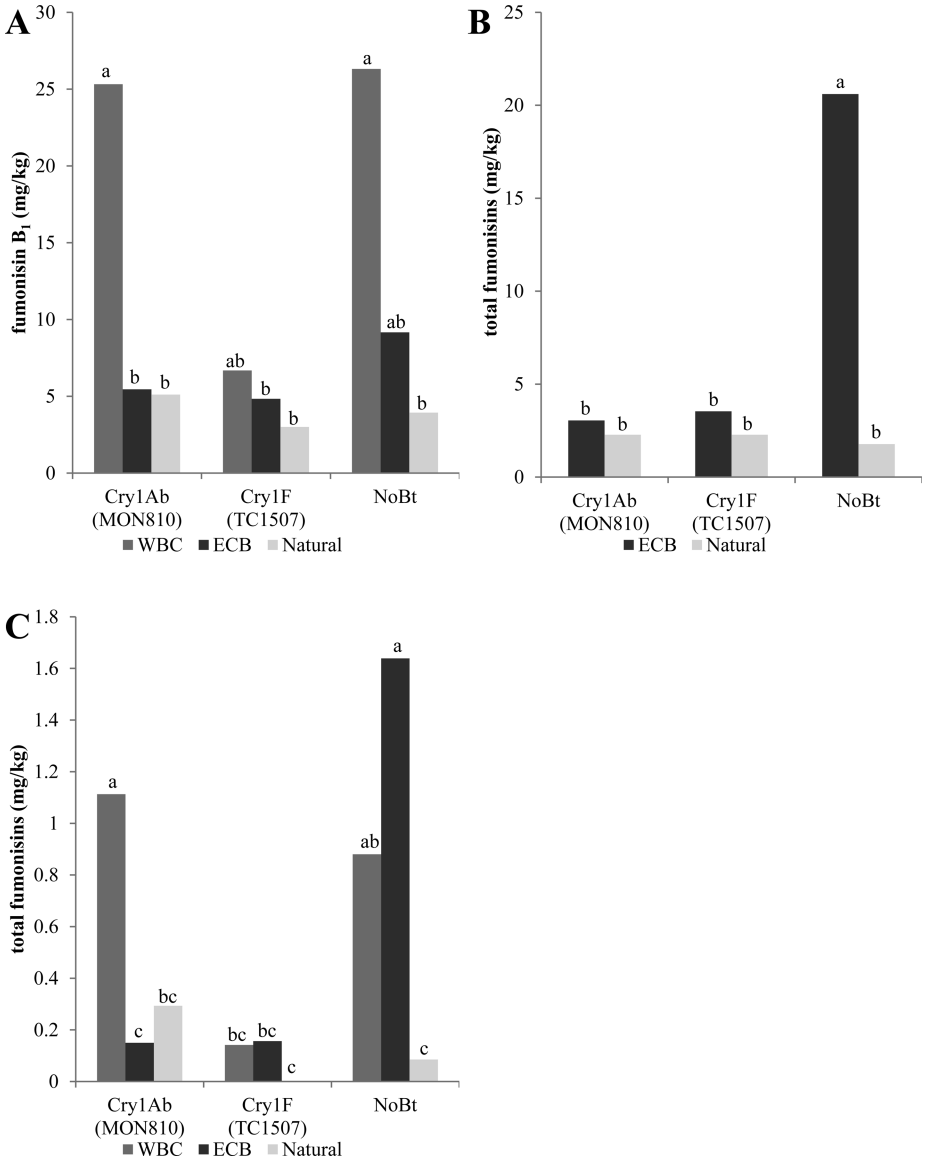

Figure 4. Fumonisin contamination in maize (mg/kg) as determined by ELISA. FB_1 contamination, **1**, is depicted for (A) 2007; total fumonisin contamination (FB_1 + FB_2 + FB_3), **1**−**3**, is depicted for (B) 2008 and (C) 2009. Data are means of five replications each of two hybrids. Letters denote significant differences ($P < 0.05$) between hybrid/insect infestations within years.

nations of total fumonisin levels (FB_1 + FB_2 + FB_3), **1**−**3**, in 2008−2009 ranged in samples from 0 to 48 mg/kg with a mean of 2.55 mg/kg. 109 samples had total fumonisin levels below 2 mg/kg, 20 samples were between 2 and 4 mg/kg, and 21 samples exceeded 4 mg/kg. ANOVA analysis revealed significant differences between years of the study ($F_{1,132}$ = 51.43 and $P < 0.0001$) with mean fumonisin levels higher in 2008 (5.59 mg/kg) than 2009 (0.50 mg/kg). In 2008, hybrid, insect infestation treatment, and their interaction were all significant factors contributing to the severity of total fumonisin contamination as determined by ELISA ($F_{2,50}$ = 4.31 and P = 0.0187, $F_{1,50}$ = 17.47 and $P = 0.0001$, $F_{2,50}$ = 5.15 and P = 0.0092, respectively) and likewise, in 2009 ($F_{2,77}$ = 15.55 and $P < 0.0001$, $F_{2,77}$ = 14.24 and $P < 0.0001$, $F_{4,77}$ = 8.11 and $P <$ 0.0001, respectively).

Total fumonisin levels as determined by HPLC ranged in individual samples from LOD to 34.87 mg/kg with a mean and standard deviation of 1.28 ± 3.19 mg/kg. A total of 197 samples had total fumonisin levels below 2 mg/kg, 17 samples were between 2 and 4 mg/kg, and 13 samples exceeded 4 mg/kg. Fumonisin contamination in maize grain (as determined by HPLC) was highest in 2008 (3.22 mg/kg), followed by 2010 (0.57 mg/kg), and lowest in 2009 (0.40 mg/kg) (Table 3).

Hybrids susceptible to WBC, namely, Cry1Ab and non-*Bt* hybrids, were significantly more contaminated with FB_1 than Cry1F hybrids which expressed insecticidal proteins against this insect. In 2007, ELISA results showed that Cry1F hybrids had less FB_1 contamination than Cry1Ab (P = 0.0423) or non-*Bt* hybrids (P = 0.0154). WBC infested plots had the highest mean FB_1 content in 2007 (19.44 mg/kg), greater than either ECB (6.48 mg/kg, P = 0.0019) or naturally infested plots (4.01 mg/kg, $P < 0.0001$) (Figure 4A). ECB infestation exacerbated total fumonisin contamination in comparison to naturally infested plots in 2008 ($P = 0.0001$ and $P = 0.0005$ for ELISA and HPLC, respectively). In 2008, non-*Bt* hybrids had higher levels of contamination than Cry1Ab hybrids or Cry1F hybrids. Among all hybrid/insect combinations in 2008, ECB infested non-*Bt* hybrids suffered this highest level of fumonisin

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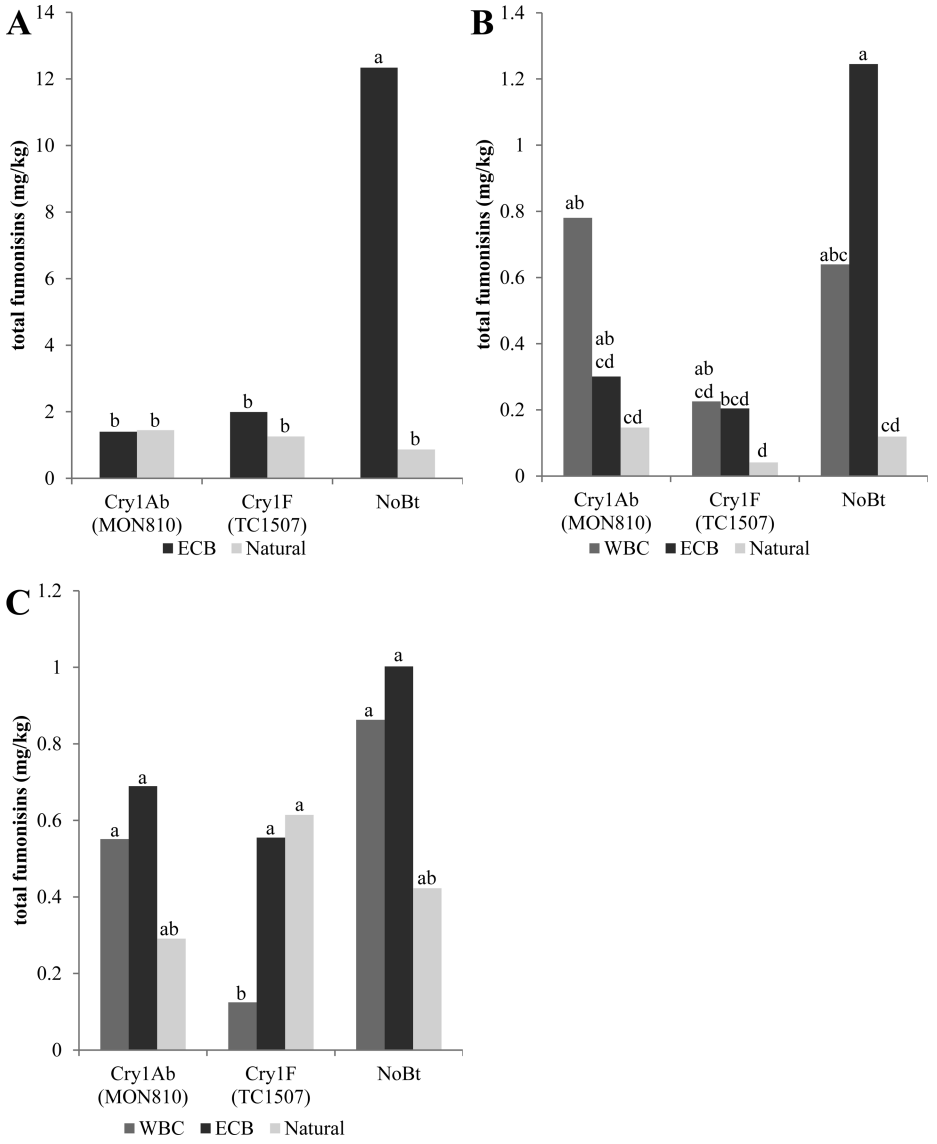

Figure 5. Fumonisin contamination, **1**−**3**, in maize (mg/kg) as determined by HPLC for (A) 2008, (B) 2009, and (C) 2010. Data are means of five replications each of two hybrids. Letters denote significant differences ($P < 0.05$) between hybrid/insect infestations within years.

contamination ($P < 0.0001$ to $P = 0.0062$) (Figure 4B and Figure 5A). In 2009, plots infested with WBC or ECB suffered higher levels of fumonisin contamination than naturally infested plots ($P < 0.0001$ and $P = 0.0012$, respectively (ELISA); $P < 0.0001$ and $P = 0.0002$, respectively (HPLC)). Cry1F hybrids in 2009 had less fumonisin contamination than Cry1Ab ($P = 0.0003$ by ELISA, $P = 0.0064$ by HPLC) or non-*Bt* hybrids ($P < 0.0001$ by ELISA, $P = 0.0005$ by HPLC) (Figure 4C and Figure 5B).

ELISA was not performed in 2010; HPLC results show that Cry1F hybrids were less contaminated than non-*Bt* hybrids ($P = 0.0101$) while Cry1Ab hybrids suffered an intermediate level of contamination relative to these, which did not differ from Cry1F or non-*Bt* hybrids. Averaged over all hybrids 2010, fumonisin contamination was the most severe in ECB infested plots, least in naturally infested plots ($P = 0.0146$), and WBC did not differ from either of these treatments. Cry1F plots infested with WBC in 2010 suffered the lowest levels of fumonisin contamination, less than either non-*Bt* or Cry1Ab plots infested with WBC ($P = 0.0002$ and $P = 0.0065$, respectively), or Cry1F plots subject to ECB or natural insect infestation treatment ($P = 0.0224$ and $P = 0.0277$, respectively) (Figure 5C). Overall, hybrids with *Bt* genes yielded grain with less kernel injury, *Fusarium* ear rot, and fumonisins compared to non-*Bt*, near-isogenic hybrids. Expression of *Bt* genes *cry1F* and *cry1Ab* in maize hybrids prevented insect feeding, thereby reducing the capability of toxigenic fungi to colonize the ear and produce mycotoxins. As a result, they experienced reduced contamination with fumonisins, compared to non-*Bt* hybrids, when grown under conditions of insect infestation. Total fumonisin contamination in maize obtained from *Bt* hybrids measured in 2008−2010 was consistently below FDA guidance levels.[31] In 2008, non-*Bt* grain would have suffered market limitations as a result of its fumonisin contamination, which exceeded 6 mg/kg as determined by HPLC and 11 mg/kg by ELISA.

In addition to the prevention of insect feeding by *Bt* maize that can result in reduced fumonisins, recent evidence also points to direct effects of *Bt* gene expression on fumonisin-producing *Fusarium* species. Rocha et al.[32] and Reis et al.[33] found that expression of genes in the fumonisin biosynthesis pathways was reduced when *F. verticillioides* fungus was grown

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on maize kernels which express either Cry1Ab[32] or Cry1F[33] as compared with near-isogenic controls. In the current study, hybrids expressing Cry1Ab and Cry1F had reduced fumonisin contamination when exposed to pest pressures for which they expressed insecticidal proteins. Cry1F hybrids had the lowest fumonisin levels in 2007, 2009, and 2010, the three years in which WBC insect infestation was used. In all years of the study, Cry1F and Cry1Ab performed similarly under ECB infestation. Under nontarget pest pressures, *Bt* hybrids did not display a significant advantage over non-*Bt* hybrids. The rise of pests vying for maize as a secure food source will increase the need for planting hybrids which target a broader spectrum of pests. This will aid in reducing kernel injury and subsequently reducing toxigenic fungi colonization and mycotoxin accumulation in maize hybrids.

HPLC versus ELISA Results. There was a strong correlation (r = 0.95, P < 0.0001, N = 138) between total fumonisin results of HPLC and ELISA methods obtained for 2008−2009, the two years of the study for which both methods were used (Figure 6). ELISA results were, on average, higher than HPLC

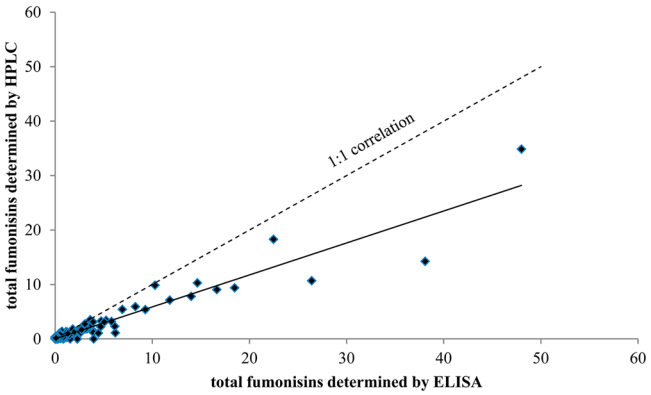

Figure 6. Correlation between total fumonisin concentration (FB_1 + FB_2 + FB_3) (mg/kg), **1**−**3**, determined by HPLC and ELISA (n = 138). Data are from 2008 and 2009.

results for the same sample, but the difference was not constant. Fumonisin contamination as determined by ELISA was correlated with insect injury (R = 0.52, P < 0.0001) and *Fusarium* ear rot (R = 0.70, P < 0.0001). These correlations were weaker when HPLC results were used to determine correlations with both insect injury (R = 0.47, P < 0.0001) and *Fusarium* ear rot (R = 0.52, P < 0.0001). Statistical significance of hybrid and insect infestation effects were generally the same for both HPLC and ELISA methods except for one case, a significant interaction effect in the 2009 ELISA data which was not significant for the HPLC data (Figures 4B and 5A, and Figures 4C and 5B). In this particular year, however, means calculated by hybrid-insect treatment combinations did not exceed 2 mg/kg for either method; it is, therefore, unlikely that market implications would have arisen from this discrepancy. Ghali et al.[34] also reported fumonisin contamination in maize by both HPLC and direct-competitive ELISA. In all commodities tested, ELISA gave more fumonisin-positive samples than HPLC. For samples detected as positive by both methods they found a significant coefficient of determination, r^2 = 0.978 (N = 25). Adoption and use of rapid test kits like ELISA in commercial settings is valuable to ensure that the mycotoxin contamination does not exceed market needs imposed by end-users. It may be appropriate to apply a correction factor to results obtained by rapid test kits, if their results differ from a validated analytical technique by a predictable factor. It is crucial, however, to use these techniques in the appropriate manner, ideally with regular check samples performed with validated analytical techniques.

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